University of Alberta

QUIESCENT HSV-1 GENOMES ARE INACCESSIBLE TO THE TRANSCRIPTION AND DNA REPLICATION MACHINERY

by

©

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ABSTRACT

Athough HSV-1 replicates freely in most tissue culture cells, mutant viruses that are incapable of expressing immediate-early (IE) genes are retained by cells in a quiescent, non-replicating state. This repression can be reversed by the provision of the HSV-1 IE protein ICP0, and ICP0 mutant viruses are incapable of reactivating a quiescent genome. However, it has not previously been shown that productive infection of an ICP0 mutant occurs in cells that harbour the quiescent viral genome.

We found that an ICP0 mutant is capable of overcoming an antiviral response triggered by infection of a quiescent mutant virus; however, this productive superinfection does not lead to reactivation of the quiescent genome. These results confirm that quiescent genomes remain silent despite the presence of all the necessary transcription and replication machinery. This indicates that the quiescent viral genome is converted into an inaccessible state or is in an inaccessible place.

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LIST OF ABBREVIATIONS

A	Adenine
C	Cytosine
C-	Carboxy
CBP	Creb binding protein
CENP	Centromeric proteins
ChIP	Chromatin immunoprecipitation assays
CK	Casein kinase
DNA	Deoxyribonucleic acid
DNA-PKcs	DNA-dependent protein kinase
ds	Double-stranded
Ds	*Discosoma sp.*
E	Early
EGFP	Enhanced green fluorescent protein
eIF	Eukaryotic initiation factor
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FISH	Fluorescent in situ hybridization
g	Glycoprotein
G	Guanine
GAS	Gamma-activated sequence
HAUSP	Herpesvirus associated ubiquitin specific protease
HCF	Host cell factor
HCMV	Human cytomegalovirus
HDAC	Histone deacetylase
HECT	Homologous to the E6-AP carboxyl terminus
HEL	Human Embryonic Lung
HIgR	Herpesvirus immunoglobulin-like receptor
HMBA	Hexamethylene bisacetamide
hnRNP	Heterogeneous nuclear ribonucleoprotein
HMG1	High mobility group protein 1
HP1	Heterochromatin protein 1
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
Hve	Herpesvirus entry protein
ICP	Infected cell protein
IE	Immediate-early
IFA	Indirect-immunofluorescence assay
IFN	Interferon
IRFs	Interferon response factors

ISGs	Interferon stimulated genes
ISREs	IFN-stimulated regulatory elements
JAKs	Janus kinases
K	Lysine
kb	Kilo-base pair
L	Late
LATs	Latency associated transcripts
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
mRNA	Messenger RNA
mt	Mutant
N-	Amino
ND10	Nuclear domain 10
NDV	New-Castle disease virus
NLS	Nuclear localization signal
NK	Natural killer cell
OAS	2'5'-Oligoadenylate synthetase
Oct-1	Octamer transcription factor 1
Ori	Origin of replication
PAA	Phosphonoacetic acid
PKR	Protein-kinase R
PML	Promyelocytic leukemia
RING	Really interesting new gene
RNA	Ribonucleic acid
RNAP II	RNA polymerase II
ss	Single-stranded
STATs	Signal transducers and activators of transcription
STD	Sexually transmitted disease
SUMO-1	Small ubiquitin-like protein
SV	Simian virus
T	Thymidine
TAF	Transcription activating factor
TAP	Transporter associated with antigen processing
TBP	TATA-binding protein
TF	Transcription factor
TK	Thymidine kinase
TNF	Tumour necrosis factor
TSA	Trichostatin A
U_L	Unique long
U_S	Unique short
USP	Ubiquitin specific protease
vhs	Virion-associated host shutoff protein
VP	Virion protein

VSV	Vesicular stomatitis virus
wt	Wild-type

CHAPTER 1 : INTRODUCTION

1.1 Overview of Herpesviruses

Herpesviridae is a family of large, DNA viruses affecting both animals and humans. All members of the herpesvirus family are capable of establishing a latent infection in their host and share the following structural characteristics: an envelope with glycoprotein spikes, amorphous tegument layer, and an icosahedral capsid encasing their linear, double-stranded DNA genome (Roizman & Pellett, 2001). To date, 9 herpesviruses capable of infecting humans have been identified. Diseases caused by these viruses can be as minor as an irritating skin lesion, or severe as encephalitis resulting in death.

The *Herpesviridae* family is subdivided into *Alphaherpesvirinae, Betaherpesvirinae, and Gammaherpesvirinae* (Roizman *et al.*, 1973). These divisions are based on factors such as host range, growth rate, and the cell-type where latency is established. The herpes simplex viruses type 1 and type 2 are members of the *Alphaherpesvirinae* subfamily. These viruses typically have a wide host range, destroy infected cells, spread rapidly in culture, and are able to establish latency in sensory ganglia (Roizman & Pellett, 2001). *Betaherpesvirinae* members, such as cytomegalovirus, generally have a restricted host range, grow slowly in culture without destroying infected cells, and establish latency in tissues such as kidney and secretory glands (Roizman & Pellett, 2001). Members of *Gammaherpesvirinae,* such as Epstein-Barr virus, often have specificity for either T or B lymphocytes, and establish latency in lymphoid tissue (Roizman & Pellett, 2001).

1.2 Introduction to herpes simplex viruses

As far back in history as ancient Greece, people have suffered from and documented herpes simplex virus infections (Wildly, 1973). The two herpes simplex viruses, type 1 (HSV-1) and type 2 (HSV-2), most commonly cause inflamed skin lesions. Their name, herpes, which means to creep or crawl, comes from the characteristic formation of these lesions (Beswick, 1962).

1.2.1 Pathogenesis

HSV is spread strictly from human-to-human (there is no animal reservoir) by contact with virus filled secretions. The virus can infect mucosal tissue or enter through breaks in the skin. HSV-1 infections primarily affect the upper body, especially mucosa of the lips, while HSV-2 primarily infects genital regions (Nahmias & Dowdle, 1968). In the primary infection, HSV infects epithelial cells and replicates. The progeny can then infect sensory neurons that innervate the innoculated tissue. Following innoculation and replication of HSV-1 on the mucosal tissue of the lips, the virus infects the trigeminal ganglion (Whitley, 2001). HSV-2 is transmitted through genital contact and after replicating in the genital regions, the virus colonizes the sacral ganglia (Whitley, 2001). HSV replicates in the sensory ganglia (Cook & Stevens, 1973) for a few days before establishing a latent state during which the viral genome resides in the nucleus of sensory neurons as a circular episome and no viral proteins are produced (Whitley, 2001). Conditions such as stress, hormonal imbalance, or exposure to ultraviolet light can cause the virus to reactivate, undergo lytic replication, and reinfect epithelial cells—leading to recurrent lesions containing infectious virus. HSV can be transmitted to a new host either from lesions or from asymptomatic shedding (Mertz *et al.*, 1988, Wald *et al.*, 2000).

1.2.2 Epidemiology

HSV is found throughout the world. Many factors, such as age, race, country, and socioeconomic status influence the prevalence of HSV-1 and HSV-2 infections (Whitley, 2001). The incidence of HSV-1 infection tends to increase linearly with age. Commonly, 40% of people are infected by the age of 15 with infection usually occurring during childhood. This number increases to 60-90% by age 70 (Smith & Robinson, 2002) with about 33% of people suffering from recurrent infections (Whitley, 2001). An important factor for the increased prevalence of HSV-1 infections in areas of lower socioeconomic status is that, because there is more crowding, there is an increased likelihood of coming in contact with the virus (Whitley, 2001).

The prevalence of HSV-2 infection shows a pattern typical for a sexually transmitted disease (STD). Before the age of 12 infection is negligible, but during sexually active years, the prevalence tends to increase and then plateau around the age of 40 (Smith & Robinson, 2002). Thirty years ago, rates of HSV-2 infection collected from STD clinics showed prevalence rates from 0.002% to 7.0% (Duenas *et al.*, 1972, Naib *et al.*, 1973). More recently, in the U.S., HSV-2 seroprevalence is about 22% overall (Fleming *et al.*, 1997). As expected of a STD, the more sexual partners, the greater chance of infection (Rawls *et al.*, 1976). HSV-2 infection can reach as high as 80% in populations with high-risk sexual behavior (Smith & Robinson, 2002).

1.2.3 Clinical Manifestations and Immune Response

The most common disease caused by HSV-1 is facial herpes labialis (or cold sores) affecting the mucosal tissue of the lips. Primary HSV-1 infection of this area is often asymptomatic; however, a variety of symptoms can also be experienced including vesicular or ulcer lesions, sore throat, fever and malaise

(Whitley, 2001). Recurrent cold sores are preceded by a painful, burning, itching sensation called a prodrome that is usually gone within 6 hrs (Spruance *et al.*, 1977). This sensation is likely caused by replication of the virus in sensory nerve endings and the epidermis (Huff *et al.*, 1981). Twenty-four to fourty-eight hrs after the prodrome, an average of 3-5 vesicles form along the borders of the lips. The vesicular fluid, between the epidermis and dermal layer, is full of virus, cell debris and inflammatory cells. After 48 hrs the lesions become pustular, then crust over. Within 8-10 days of disease onset, healing is usually complete with scarring occurring only in rare cases of very frequent recurrent infection (Whitley, 2001).

In contrast to the often asymptomatic, primary HSV-1 infection, the most severe symptoms are experienced during the primary HSV-2 infection. Vesicular, pustular and ulcer lesions develop on genital regions and last for an average of 3 weeks (Whitley, 2001). Women experience much more severe symptoms than men and have painful lesions that form bilaterally on the vulva and commonly on the cervix as well. Men develop an average of 6-10 vesicular lesions on the glans penis or penile shaft (Whitley, 2001). Other symptoms can include fever, malaise and dysuria. Recurrent genital herpes are preceded by a painful prodrome (Luby *et al.*, 1984), but the symptoms are less severe, and of a shorter duration than for a primary infection. Symptoms last for 7–10 days and, for women, generally involve irritation of the vulva, while men develop 3–5 vesicles on the penis (Adams *et al.*, 1976).

Although HSV primarily affects mucosal tissue, if the immune system has been sufficiently compromised, it is capable of infecting any tissue in the human body. Medical and dental personnel, with constant hand washing possibly leading to abrasions, are particularly prone to an HSV infection of the digits called herpetic whitlow (Rosato *et al.*, 1970). Severely immunocompromised individuals, such as transplant recipients, can develop severe progessive disease involving the respiratory or gastrointestinal tracts

(Montgomerie *et al.*, 1969). Babies infected *in utero* are at risk of disseminated infections involving multiple organs such as liver, adrenals or even the brain (Whitley, 2001). Encephalitis is a particularly devastating HSV infection: virus replication in the central nervous tissue often causes death. Luckily, this is a rare occurrence estimated to be 1 in 200,000 (Whitley, 2001).

The important host defenses against HSV include antibodies, macrophages, natural killer (NK) cells, T cells, and lymphokine responses (Whitley, 2001). The antiviral effects of IFN will be discussed in detail in a later section. Antibodies to HSV appear 2-6 weeks post-infection and persist for life (Kohl *et al.*, 1982). A mixture of HSV-specific antibodies and complement, or a mixture of antibodies with monocytes, macrophages, or killer lymphocytes can lyse HSV-infected cells *in vitro* (Rouse, 1985), illustrating their roles in limiting HSV infection.

1.3 HSV-1 Virion

The HSV-1 virion is 150-200 nm in diameter (Roizman & Knipe, 2001) and consists of an envelope, tegument layer, capsid and genome.

1.3.1 Envelope

The HSV-1 lipid membrane envelope is acquired from a post-ER cytoplasmic compartment of the host cell (Skepper *et al.*, 2001, Whiteley *et al.*, 1999). The envelope contains at least 10 viral glycoproteins (gB, gC, gD, gE, gG, gH, gI, gK, gL, and gM) and at least two nonglycosylated intrinsic membrane proteins (U_L20 and U_S9) (Roizman & Knipe, 2001). The glycoproteins, which are involved in entry of the virus into the host cell, are nonrandomly distributed and appear as spikes projecting from the virion envelope (Stannard *et al.*, 1987).

1.3.2 Tegument

Between the capsid and the envelope of HSV-1 is the often asymmetrical tegument layer that contains varying amounts of at least 20 different viral proteins (Subak-Sharpe & Dargan, 1998). Some of these proteins are important for the initiation of viral gene expression, for example, the transcription factor virion protein (VP) 16 (Campbell *et al.*, 1984). Others make the host cell environment more conducive to viral growth. Examples of these are ICP0, which will be discussed in detail, the virion-associated host shutoff protein (vhs) which degrades cellular and viral mRNAs (Kwong & Frenkel, 1987, Oroskar & Read, 1987), and U_S11, which is an RNA binding protein that inactivates protein-kinase R (PKR), preventing interferon (IFN) induced translational arrest (Cassady *et al.*, 1998).

1.3.3 Capsid

The approximately 125 nm diameter HSV-1 capsid (Steven & Spear, 1996) encases and protects the viral genome. The capsid shell is composed of 162 capsomeres which are arranged into T=16 icosahedral symmetry (Roizman & Knipe, 2001) with 12 pentons on the vertices, and 150 hexons on the edges and faces. The major structural protein of the pentons and hexons is VP5, with the outer tips of the hexons formed by VP26 (Steven & Spear, 1996). Triplexes, composed of one molecule of VP19C and two molecules of VP23, connect the capsomeres (Newcomb *et al.*, 1993).

1.3.4 Genome

The packaged HSV-1 genome is predominantly linear, double-stranded DNA (Kieff *et al.*, 1971), however, its precise arrangement in the capsid is unclear. Early evidence indicated a toroid form (Furlong *et al.*, 1972), but more recent experiments have failed to confirm this (Subak-Sharpe & Dargan, 1998). The HSV-1 genome is approximately 152 kbp in length, encodes at least 80 viral proteins (Roizman & Knipe, 2001), and has a G+C content of 68% (Kieff *et al.*, 1971). The genome consists of covalently linked unique long (U_L) and unique short (U_S) segments each flanked by inverted repeats (Wadsworth *et al.*, 1975), resulting in two copies of each of the genes located in the inverted repeat regions. The long and short segments can invert relative to each other, giving rise to 4 linear isomers, equal ratios of which can be found in any given population (Delius & Clements, 1976, Hayward *et al.*, 1975). A common naming system for HSV genes and gene products is by their relative order from left to right in the U_L or U_S segments of the genome (e.g. U_L1 or U_S1). Other naming systems include infected cell protein (ICP) number, virion protein (VP) number, and molecular weight. Although many HSV proteins are referred to in the literature by more than one name, here, for simplicity's sake, only one will be mentioned.

1.4 HSV-1 Lytic infection

Lytic infection begins with attachment of the virus particle to cell surface receptors followed by fusion of the viral envelope to the cellular membrane. The tegument and nucleocapsid are released into the cytoplasm and transported to nuclear pores where the viral DNA enters. Viral gene expression then occurs in a temporally regulated program: immediate early (IE) to early (E) to late (L). Following viral DNA replication and L gene expression, the viral genome is packaged into pre-assembled capsids. Through a series of envelopment and de-envelopment steps, the nucleocapsid exits the nucleus, acquires a tegument layer and envelope in the cytoplasm, and is released from the cell (Roizman & Knipe, 2001).

1.4.1 Attachment and Entry

In the first stage of entry, initiation of attachment, gC interacts with cell surface heparan sulfate moieties (Shieh *et al.*, 1992); however, gC is not absolutely essential for entry (Heine *et al.*, 1974). In the next stage of entry, stabilization of attachment, gD interacts with cellular receptors from three different families. A cellular receptor from the tumour necrosis family (TNF) is the herpesvirus entry (Hve) protein HveA (Montgomery *et al.*, 1996). Receptors from the immunoglobulin familly include HveB (Warner *et al.*, 1998), HveC (or nectin-1α) (Geraghty *et al.*, 1998), and herpesvirus immunoglobulin-like receptor HIgR (or nectin-2α) (Cocchi & al., 1998). 3-O-sulfated heparan sulfate is a member of the third group of cellular receptor families (Shukla *et al.*, 1999). For the third stage of entry it is widely accepted that the nucleocapsid enters the cell following fusion of the viral envelope to the host cell plasma membrane; however, the mechanism for fusion is unknown. Fusion of the membranes requires gD (Ligas & Johnson, 1988), gB (Sarmiento *et al.*, 1979), and the gH/gL heterodimer (Forrester *et al.*, 1992). The entire process of attaching, binding to co-receptors and penetrating the cell takes only minutes (Huang & Wagner, 1964). The nucleocapsid and tegument layer enter the cytoplasm and the nucleocapsid is transported to the nuclear pore by microtubules (Sodeik *et al.*, 1997). Some tegument proteins, for e.g. VP16, are transported to the nucleus, while others, for e.g. vhs and U_S11, stay in the cytoplasm. Once at the nuclear membrane the nucleocapsid associates with nuclear pore complexes (Batterson *et al.*, 1983) where the viral genome is released into the nucleus while empty capsids remain associated with the nuclear pore (Batterson *et al.*, 1983).

The genome of HSV, as well as other DNA viruses, localizes to the periphery of nuclear domain 10 (ND10) structures, also known as promyelocytic leukemia (PML) bodies, following infection (Ishov & Maul, 1996, Maul *et al.*, 1996). There are 5-20 ND10 per nucleus, each about 0.5 µm in diameter (Maul, 1998, Sternsdorf *et al.*, 1997a), and recent evidence indicates that the incoming viral genome may even recruit cellular ND10 proteins (Everett *et al.*, 2004b). PML is the major organizing component of ND10 (Ishov *et al.*, 1999, Zhong *et al.*, 2000). Other ND10 components recruited by PML include Daxx, Creb binding protein (CBP), and Sp100 (Negorev & Maul, 2001). It is still under debate as to whether ND10 are depositions of cellular factors that are helpful for viral gene expression (such as transactivating factors), or if they are part of an intranuclear defense mechanism that is protective against viral infection (Everett *et al.*, 2004b). The role of ND10 as a possible IFN-stimulated defense mechanism targeted by ICP0 will be discussed in later sections (1.4.2.5.1 and 1.7.2).

1.4.2 IE gene expression

IE gene transcription is thought to take place at the periphery of ND10 (Maul *et al.*, 1996) and maximum expression is seen 2-4 hrs post-infection for a multiplicity of infection (MOI) of 10-20 (Honess & Roizman, 1974). The defining feature of IE genes is that their transcription does not require prior viral protein synthesis. Transcription of viral genes is mediated by host RNA polymerase II (RNAP II) (Alwine *et al.*, 1974, Constanzo *et al.*, 1977) in conjunction with viral proteins to enhance transcription of certain genes. The viral transcriptional activator VP16 stimulates the expression of the immediate early genes. In order to do this, VP16 associates with cellular proteins;

octamer transcription factor 1 (Oct-1), host cell factor (HCF), and the transcription factors TFII-B and TFII-D (Katan *et al.*, 1990, Kristie & Sharp, 1990, Lin, 1991, Stern & Herr, 1991, Stringer *et al.*, 1990, Xiao & Capone, 1990). Once in the cytoplasm, VP16 is released from the tegument and binds HCF (Katan *et al.*, 1990, Kristie & Sharp, 1990, Xiao & Capone, 1990) which transports VP16 to the nucleus (La Boissiere *et al.*, 1999, Triezenberg *et al.*, 1988). The viral IE genes each have TAATGArATT sites within a few hundred base pairs upstream of the cap site (Gaffney *et al.*, 1985, Mackem & Roizman, 1980, Mackem & Roizman, 1982). Oct-1 binds to this consensus sequence and, once bound, is able to recruit VP16/HCF. The bound Oct-1/VP16/HCF complex attracts and positions host RNAP II and, together with other cellular transcription factors, promotes IE gene transcription (Kristie *et al.*, 1989, Kristie & Roizman, 1987, O'Hare & Gooding, 1988, Preston *et al.*, 1988). VP16 transactivation requires the acidic carboxyl-terminal residues (Triezenberg *et al.*, 1988) and is not critical for gene expression in rapidly dividing cells (Spector *et al.*, 1991), but may be more important in resting cells.

Commonly, the 5 IE gene products (ICP47, ICP22, ICP27, ICP4 and ICP0) are referred to based on their infected-cell-protein (ICP) designation. In this system, proteins that were found in infected cells, but not uninfected cells were numbered based on their decreasing apparent molecular weight on denaturing gels (Honess & Roizman, 1973).

1.4.2.1 ICP47

ICP47 is a 12 kDa protein encoded by the α47 gene (Roizman & Knipe, 2001) located in the unique short segment of the viral genome. A unique feature of ICP47 among the 5 IE gene products is that it does not have a gene regulatory function. Instead, ICP47 is involved in immune evasion which, despite being nonessential for growth in rabbit and Vero cell lines (Longnecker & Roizman, 1986) is an important aspect of viral survival in natural infection. ICP47 is a cytoplasmic protein that colocalizes and interacts with TAP (transporter associated with antigen processing) (Fruh *et al.*, 1995, Hill *et al.*, 1995). The interaction of ICP47 with TAP prevents TAP from transporting proteasome degraded antigenic peptides from the cytoplasm to the endoplasmic reticulum (ER) lumen (Fruh *et al.*, 1995, Hill *et al.*, 1995). In the absence of peptides the class I molecules are retained in the ER: preventing cytotoxic T-lymphoctye recognition and lysis of infected cells (Fruh *et al.*, 1995, Hill *et al.*, 1995, York *et al.*, 1994).

Currently, ICP47 is being investigated as a mechanism to induce local immunosuppression, thereby preventing host rejection in response to transgenes or allografts (Radosevich *et al.*, 2003). A major obstacle confronting successful xenotransplantation is host rejection. Promising experiments have shown that expression of ICP47 leads to downregulation of porcine major histocompatibility complexes (MHC) and prevents proliferation of human T cells in response to pig kidney cells (Crew & Phanavanh, 2003).

1.4.2.2 ICP22

ICP22 is a 68 kDa protein encoded by the α22 gene (Roizman & Knipe, 2001) located in the unique short segment of the viral genome. U_S1.5 is also encoded by the α22 gene and has the same sequence as the 249 C-terminal amino acids of ICP22 (Carter & Roizman, 1995). ICP22 is a nuclear protein and has two independent nuclear localization signals (Stelz *et al.*, 2002). Early in infection, ICP22 is found in small, dense nuclear bodies (Jahedi *et al.*, 1999). Following the initiation of DNA replication, and phosphorylation of ICP22 by the U_L13 viral protein kinase (Leopardi *et al.*, 1997), ICP22 localizes to transcriptional complexes containing viral DNA, RNAP II, ICP4, and other factors

(Jahedi *et al.*, 1999, Leopardi *et al.*, 1997). Late in infection ICP22, along with the U_L3 and U_L4 gene products, relocalizes to small dense nuclear bodies (Markovitz & Roizman, 2000).

ICP22 is a multifunctional regulatory protein that is required for the efficient expression of ICP0 as well as a subset of true late genes including U_S11, U_L41, and U_L38 (Purves *et al.*, 1993, Sears *et al.*, 1985). In addition to this, ICP22 may act as a repressor of IE gene expression (Prod'hon *et al.*, 1996). Although ICP22 is non-essential for growth is cell-culture lines such as Vero cells (Post & Roizman, 1981), ICP22 deletion mutants replicate poorly in primary human fibroblasts, rabbit, and rodent cells (Sears *et al.*, 1985), and have attenuated virulence when inoculated intracerebrally into mice (Roizman & Knipe, 2001).

Post-translational modifications of ICP22 include nucleotydylation by casein kinase II (Blaho *et al.*, 1993, Mitchell *et al.*, 1997) and phosphorylation by the viral protein kinases U_L13 and U_S3 (Prod'hon *et al.*, 1996, Purves *et al.*, 1993, Purves & Roizman, 1992). The similar phenotype of ICP22 and U_L13 mutants indicates the importance of U_L13 phosphorylation on the action of ICP22 (Purves *et al.*, 1993).

The exact mechanism for ICP22 function is unclear. ICP22 is known to influence the ICP0 mRNA splicing pattern (Carter & Roizman, 1996) as well as to interact with cellular proteins such as p78 (Bruni & Roizman, 1998) and p60 (a cellular protein of unknown function that also interacts with ICP0) (Bruni *et al.*, 1999). The inefficient transcription of a subset of true late genes seen for ICP22 mutants may be due to a lack of an alternatively phosphorylated form of the C-terminal domain of RNAP II (Long *et al.*, 1999, Rice *et al.*, 1995, Rice *et al.*, 1994).

A further clue for the role of ICP22 in the efficient expression of late gene products is the requirement of ICP22 for the posttranslational modification and interaction of topoisomerase IIα with a cdc2-U_L42 complex

(Advani *et al.*, 2003). ICP22 and U$_L$13 are required for the posttranslational modification and activation of cdc2, as well as the degradation of cylins A and B (Advani *et al.*, 2000a). The viral DNA polymerase processivity factor U$_L$42 replaces cyclin B as a binding partner of cdc2 (Advani *et al.*, 2001b). The cdc2-U$_L$42 complex is then able to recruit posttranslationally modified topoisomerase IIα (Advani *et al.*, 2003). As ICP22 mutants are capable of viral DNA synthesis (Sears *et al.*, 1985), it appears that the topoisomersase is recruited for a function other than viral DNA synthesis. Advani and colleagues hypothesize that topoisomerase IIα is required for untangling newly-synthesized, concatemeric viral DNA: allowing efficient transcription of late genes (Advani *et al.*, 2003).

1.4.2.3 ICP27

ICP27 is a 63 kDa multi-functional protein encoded by the U$_L$54 gene (Roizman & Knipe, 2001). The N-terminal region of ICP27 includes a nuclear localization signal (Mears *et al.*, 1995), as well as a leucine-rich nuclear export signal (Sandri-Goldin, 1998). Early times post-infection ICP27 is diffusely distributed in the nucleus, while at later times ICP27 is found in replication compartments (de Bruyn Kops *et al.*, 1998) and shuttling between the nucleus and the cytoplasm (Mears & Rice, 1998, Phelan & Clements, 1997, Sandri-Goldin, 1998, Soliman *et al.*, 1997).

ICP27 is an essential regulatory protein (McCarthy *et al.*, 1989) (Sacks *et al.*, 1985) with roles both in the activation and repression of viral gene expression. ICP27 deletion mutants have greatly decreased amounts of viral DNA, over-express E genes, have only low levels of leaky late proteins, and do not express true late gene products (McCarthy *et al.*, 1989, McMahan & Schaffer, 1990, Rice & Knipe, 1990). A subset of E genes require ICP27 for efficient expression (McGregor *et al.*, 1996, Samaniego *et al.*, 1995, Uprichard

& Knipe, 1996). The products of these E genes include viral DNA replication proteins, indicating that the reduced viral DNA synthesis seen for ICP27 mutants may result from insufficient expression of genes important for replication (Uprichard & Knipe, 1996).

There is evidence that ICP27 can regulate transcription (Jean *et al.*, 2001, Spencer *et al.*, 1997) and associate with RNA polymerase II complexes (Jenkins & Spencer, 2001, Zhou & Knipe, 2002). However, many of the effects of ICP27 on viral gene expression are at the post-transcriptional level. For example, ICP27 may enhance the expression of viral L genes with weak poly(A) signals by altering the specificity of polyadenylation machinery; stimulating 3'-end mRNA processing (McGregor *et al.*, 1996) (McLauchlan *et al.*, 1992, McLauchlan *et al.*, 1989, Sandri-Goldin & Mendoza, 1992). Also, ICP27 inhibition of host cell splicing occurs post-transcriptionally (Hardwicke & Sandri-Goldin, 1994, Hardy & Sandri-Goldin, 1994) at the level of spliceosome assembly (Lindberg & Kreivi, 2002).

Export of unspliced mRNA from the nucleus to the cytoplasm is generally very inefficient in human (and all metazoan) cells (Luo & Reed, 1999). As almost all of the transcripts expressed by HSV during lytic replication are intronless, it is important for the virus to have a mechanism of ensuring efficient export of its unspliced mRNA. The RNA binding properties of ICP27 (through an arginine-rich RGG box) (Ingram *et al.*, 1996, Mears & Rice, 1996, Sandri-Goldin, 1998) coupled with its ability to shuttle between the nucleus and cytoplasm (Mears & Rice, 1998, Phelan & Clements, 1997, Soliman *et al.*, 1997) are clear indicators of a role in the export of unspliced viral mRNAs; however, the exact mechanism is still unclear. The initial model for ICP27 mediated export of unspliced viral mRNAs was through the cellular adaptor CRM1 (Soliman & Silverstein, 2000), however CRM1 was subsequently reported to be unnecessary for ICP27-mediated export in *Xenopus laevis* oocytes (Koffa *et al.*, 2001), or for the activity of ICP27's leucine-rich NES

(Chen *et al.*, 2002). Another popular model is that ICP27 binds to and recruits Aly/REF (a component of the exon junction complex) to viral intronless mRNAs, thereby providing them with access to the TAP export pathway (Chen *et al.*, 2002, Koffa *et al.*, 2001). However, a deletion mutant incapable of the ICP27/REF interaction is not greatly inhibited on Vero cells (Lengyel *et al.*, 2002).

The interaction between ICP27 and a number of cellular proteins involved in splicing contributes to the ICP27-mediated inhibition of splicing, which may enhance export of viral transcripts. For example, ICP27 interacts with both SR proteins (essential splicing factors) and SRPK1 (a protein kinase) (Sciabica *et al.*, 2003). The interaction between ICP27 and SRPK1 is proposed to lead to the hypophosphorylation of the SR proteins thereby impairing their action in spliceosome assembly (Sciabica *et al.*, 2003). ICP27 also interacts with heterogeneous nuclear ribonucleoprotein (hnRNP) K, casein kinase 2 (CK2) (Wadd *et al.*, 1999), and p32 (Bryant *et al.*, 2000). In the presence of ICP27, a p32-hnRNPK-CK2 complex can form which, together, may be able to inhibit splicing (Bryant *et al.*, 2000). Another ICP27 interaction important for the inhibition of splicing is between ICP27 and spliceosome-associated protein 145 (SAP145), an essential splicing factor (Bryant *et al.*, 2001). ICP27 mutants that are unable to inhibit splicing have either reduced or no interaction between ICP27 and SAP145 (Bryant *et al.*, 2001).

1.4.2.4 ICP4

ICP4 is a 175 kDa protein encoded for by two copies of the $\alpha4$ gene (Roizman & Knipe, 2001) located in the inverted repeat region flanking the short segment of the genome. In infected cells ICP4 is nuclear localized and, at early times post-infection, is found diffuse throughout the nucleus (Knipe *et al.*, 1987). Recent evidence suggests that ICP4 is rapidly recruited to the viral genome following entrance into the nucleus (Everett *et al.*, 2004b). Later on in infection ICP4 is redistributed to globular replication compartments, within which it has a somewhat diffuse distribution (de Bruyn Kops *et al.*, 1998). The redistribution of ICP4 to replication compartments is dependent upon viral DNA replication (Knipe *et al.*, 1987).

ICP4 is a major regulatory protein of HSV transcription and acts as both a transcriptional activator and a repressor. The ICP4-mediated transcriptional activation of the E and L genes (Watson & Clements, 1980) is absolutely required for viral growth (DeLuca *et al.*, 1985, Dixon & Schaffer, 1980, Preston, 1979). Infection of Vero cells with an HSV-1 mutant for both copies of ICP4 leads to production of only ICP6, ICP0, ICP22, and ICP27 (DeLuca *et al.*, 1985). The mechanism by which ICP4 activates transcription is not yet fully understood, however some essential elements have been identified. ICP4 is a site-specific DNA binding protein that binds to both consensus and nonconsensus sites (Kristie & Roizman, 1986, Michael & Roizman, 1989, Michael *et al.*, 1988). In order to activate transcription, ICP4 must interact with DNA 3' to the start site (Gu & DeLuca, 1994). The regulatory region need contain only a TATA box and an initiator element for ICP4 to activate transcription (Carrozza & DeLuca, 1998). ICP4 helps the TATA binding protein (TBP)-containing general transcription factor (TFIID) bind to the promoter and the TATA box (Grondin & DeLuca, 2000). The interaction between ICP4 and TFIID requires transcription activating factor (TAF) 250, which interacts with the C-terminal region of ICP4 (Carrozza & DeLuca, 1996). Binding of TFIID to the promoter region with the help of ICP4 facilitates the formation of transcription pre-initiation complexes (Grondin & DeLuca, 2000). Formation of these higher-order DNA-protein complexes is further facilitated by high mobility group protein 1 (HMG1), possibly by the ability of this protein to bend DNA (Carrozza & DeLuca, 1998).

Similarly to its mechanism of activating expression of E and L genes, the mechanism by which ICP4 represses the expression of viral genes is not yet fully understood. Promoters that have ICP4 binding sites near the transcription start site are subject to transcriptional repression by ICP4 (Kuddus *et al.*, 1995). ICP4 binds to its own transcription start site (Muller, 1987), and therefore has a negative autoregulatory role on its own expression (Michael & Roizman, 1993, O'Hare & Hayward, 1985b). Binding of ICP4 to DNA regions near and 3' to the TATA box in the proper orientation allows the formation of a tripartite complex composed of ICP4, TBP, and TFIIB (Gu & DeLuca, 1994, Kuddus *et al.*, 1995). This interference with the formation of transcription initiation complexes inhibits transcription (Gu & DeLuca, 1994).

1.4.2.5 ICP0

ICP0 is a 110 kDa multi-functional protein encoded by the α0 gene (Roizman & Knipe, 2001) located in the inverted repeat region flanking the U_L segment of the viral genome, and is therefore present in two copies. ICP0 was difficult to identify using the ICP-system because its electrophoretic mobility varied when the composition of the denaturing gel was changed (Honess & Roizman, 1974).

1.4.2.5.1 Function of ICP0

Following infection, ICP0 localizes to the nucleus. Initial ICP0 accumulation is at ND10 but, as more accumulates, ICP0 fills the nucleus (Everett & Maul, 1994, Maul & Everett, 1994, Maul *et al.*, 1993). ICP0 is retained in the nucleus until viral DNA replication has begun (Lopez *et al.*, 2001), then moves to the cytoplasm (Kawaguchi *et al.*, 1997a, Lopez *et al.*, 2001, Van Sant *et al.*, 2001b). In HEL cells, translocation of ICP0 to the cytoplasm begins 3 hrs post-infection and is almost complete by 7-9 hrs (depending on MOI) (Van Sant *et al.*, 2001b). Translocation likely requires true late proteins as it does not occur when DNA synthesis is blocked (Van Sant *et al.*, 2001b). The binding of ICP0 to the cell cycle regulator cyclin D3 (Kawaguchi *et al.*, 1997b, Van Sant *et al.*, 2001b) is involved in export of ICP0 to the cytoplasm (Van Sant *et al.*, 1999, Van Sant *et al.*, 2001b) but the mechanism for this is unknown.

Unlike most HSV genes, ICP0 is spliced. Exons 1, 2 and 3 encode amino acids (aa) 1-19, 20-241, and 242-775 respectively (Hagglund & Roizman, 2004). The N-terminus contains a C_3HC_4 RING (really interesting new gene) finger zinc-binding motif (in exon 2) (Everett *et al.*, 1993, Everett *et al.*, 1995, Freemont *et al.*, 1991). The nuclear localization signal (NLS) maps to exon 3 (Everett, 1988). The C-terminal of ICP0 contains the ND10 localization and USP7 (also known as HAUSP or herpesvirus associated ubiquitin specific protease) binding domains (Everett, 1987, Everett, 1988) as well as a high-affinity self-interaction domain that allows the formation of dimers and oligomers (Ciufo *et al.*, 1994, Everett *et al.*, 1991a). See Figure 1.1 for a diagram of the ICP0 transcript and functional domains.

The function performed by ICP0 is likely regulated by posttranslational modifications (Advani *et al.*, 2001a): contributing to its multifunctionality. During the course of infection at least 6 different modified isoforms of ICP0 are seen (Ackermann *et al.*, 1984, Advani *et al.*, 2001a). ICP0 is nucleotidylylated by casein kinase II (Blaho *et al.*, 1993), and phosphorylated by both viral (U_L13) (Ogle *et al.*, 1997) and cellular (cdc2) (Advani *et al.*, 2000b) protein kinases.

ICP0 is a non-specific (often referred to as promiscuous) transactivator that can induce the expression of a subset of cellular genes (Hobbs & DeLuca, 1999) as well as all classes (IE, E and L) of viral promoters (Everett, 1984, Gelman & Silverstein, 1985, O'Hare & Hayward, 1985a, Quinlan & Knipe, 1985). Specific promoter sequences necessary for activation by ICP0 have not been identified (Everett *et al.*, 1991b). ICP0 and ICP4 interact with each other (Everett, 1988, Yao & Schaffer, 1994) and have a synergistic effect on the transactivation of gene expression (Everett *et al.*, 1991b). ICP0 does not bind directly to DNA (Everett *et al.*, 1991a) therefore the affect of ICP0 on transcription is likely to be indirect. It has been shown that if the RING finger is disrupted, the ability of ICP0 to transactivate gene expression is lost (Chen & Silverstein, 1992, Everett, 1987, Everett, 1988, Everett *et al.*, 1995, Lium & Silverstein, 1997).

ICP0 is an E3 ubiquitin ligase that, as mentioned earlier, contains a C_3HC_4 RING finger zinc-binding motif (Boutell *et al.*, 2002, Everett *et al.*, 1993, Everett *et al.*, 1995, Freemont *et al.*, 1991). RING finger proteins have been shown to be part of ubiquitin ligase complexes and are involved in the degradation of specific target proteins (Freemont, 2000). Proteins are targeted for 26S proteasomal degradation by the conjugation of ubiquitin chains (see Figure 1.2). Ubiquitin ligase complexes induce the accumulation of polyubiquitin chains through the action of an E1ubiquitin activating enzyme, an E2 ubiquitin conjugating enzyme, and an E3 ubiquitin ligase (reviewed in (Hershko & Ciechanover, 1998, Jackson *et al.*, 2000)). E3 ubiquitin ligase enzymes promote the transfer of ubiquitin from E2 to the substrate protein (Jackson *et al.*, 2000, Joazeiro *et al.*, 1999, Lorick *et al.*, 1999). There are two broad classes of E3 ubiquitin ligases. The first class contain a HECT (homologous to the E6-AP carboxyl terminus) related domain (Schwartz & Hochstrasser, 2003). The second, more abundant, class contain a RING finger domain (Schwartz & Hochstrasser, 2003). The importance of the RING finger domain for the activity of ICP0 is illustrated by the finding that RING finger mutants have a similar phenotype to ICP0 null mutants (Chen & Silverstein, 1992, Everett, 1989, Everett *et al.*, 1999c, Hagglund & Roizman, 2003, Lium & Silverstein, 1997, Van Sant *et al.*, 1999). ICP0 colocalizes with and induces the accumulation of conjugated ubiquitin (Everett, 2000a, Parkinson & Everett, 2001) and promotes the degradation of cellular proteins (described below) (Everett *et al.*, 1999a, Everett *et al.*, 1998a, Lees-Miller *et al.*, 1996, Lomonte *et al.*, 2001, Parkinson & Everett, 2000, Parkinson *et al.*, 1999) in a RING finger domain dependent manner.

Inactivation of the ubiquitin-proteasome pathway inhibits ICP0-mediated stimulation of the lytic cycle (Everett *et al.*, 1998a). ICP0 has been shown to interact with proteasomes (Everett *et al.*, 1998a, Everett *et al.*, 1997) and to increase the interaction of cdc34 (an E2 ubiquitin-conjugating enzyme) with proteasomes (Van Sant *et al.*, 2001a). Use of the proteasome inhibitor MG132 causes ICP0 to remain associated with proteasome subunits (Lopez *et al.*, 2001, Van Sant *et al.*, 2001a) and abrogates the ICP0 induced degradation of cellular proteins (Everett *et al.*, 1998a).

The interaction between ICP0 and USP7 (Everett *et al.*, 1999c, Everett *et al.*, 1997, Meredith *et al.*, 1995, Meredith *et al.*, 1994) may further enhance the degradation of targeted cellular proteins. USP7 is a novel member of the ubiquitin-specific protease family that localizes in or close to ND10 (Everett *et al.*, 1997). USP enzymes protect proteins from degradation by cleaving ubiquitin from the targeted protein (Wilkinson, 1995). It is hypothesized that sequestration of USP7 by ICP0 from newly ubiquitylated substrates may increase the efficiency of degradation (Hagglund & Roizman, 2004). Recently, it has been shown that the interaction between ICP0 and USP7 protects ICP0 from auto-ubiquitination and subsequent degradation (Canning *et al.*, 2004).

There is increasing evidence to support that an important role of ICP0 is to promote the proteasomal degradation of cellular proteins that may hinder viral infection. A prime example of this is the ICP0-mediated disruption of ND10 structures (Everett & Maul, 1994, Maul & Everett, 1994, Maul *et al.*, 1993). The disaggregation of ND10 following HSV infection is a result of the degradation of its main organizing protein PML (Ishov *et al.*, 1999, Zhong *et* *al.*, 2000). The antiviral affects of PML will be discussed in section 1.7.2. SUMO-1 (small ubiquitin-like protein) modification is important for the integrity of ND10 structures (Duprez *et al.*, 1999, Kamitani *et al.*, 1998, Muller *et al.*, 1998, Sternsdorf *et al.*, 1997b), and both PML and Sp100 are covalently modified by SUMO-1 in ND10 (Chelbi-Alix & de The, 1999, Kamitani *et al.*, 1998, Parkinson & Everett, 2000, Sternsdorf *et al.*, 1997b). ICP0 promotes the degradation of SUMO-1 or PIC1 (a ubiquitin-homology family protein) conjugated isoforms of PML (Everett *et al.*, 1998a) and Sp100 (Chelbi-Alix & de The, 1999, Parkinson & Everett, 2000). Other ND10 components targeted for degradation by ICP0 are still being researched. As previously mentioned, ICP0 binds to USP7, a member of the ubiquitin-specific protease family associated with ND10 (Everett *et al.*, 1997). It has been proposed that ICP0 binding to USP7 allows USP7 to cleave SUMO-1 from PML: allowing for its subsequent ubiquitination and degradation (Everett *et al.*, 1998a). ICP0-mediated disruption of ND10 requires the RING finger domain (N-terminal) and the C-terminal ND10 localization sequence (Everett & Maul, 1994, Maul & Everett, 1994) and is proteasome-dependent (Everett *et al.*, 1998a, Everett *et al.*, 1995).

In addition to ND10 components, ICP0 mediates the degradation of other cellular proteins. ICP0 leads to the degradation of centromeric proteins CENP-C and CENP-A (Everett *et al.*, 1999a, Lomonte & Everett, 1999). Transfection of ICP0 can prevent progression from G1 to S phase and block cells at pseudo-prometaphase (an unusual stage of mitosis). The block in pseudo-prometaphase correlates with the degradation of CENP-C (an important component of kinetochores) and is induced by ICP0 in a proteasomal-dependent manner (Everett *et al.*, 1999a, Lomonte & Everett, 1999). The zinc finger of ICP0 binds to kinetochores and a mutation in the region required for binding USP7 also prevents ICP0 binding to kinetochores (Everett *et al.*, 1999a). ICP0 also causes degradation of the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) (Lees-Miller *et al.*, 1996, Parkinson *et al.*, 1999). This involves the proteasome pathway, but doesn't require ICP0 binding to USP7 (Parkinson *et al.*, 1999).

In addition to its previously described activities, the interaction between ICP0 and a number of cellular proteins indicates further roles for ICP0 during infection. ICP0 colocalizes and interacts with the class II histone deacetylase (HDAC) 4 (Lomonte *et al.*, 2004). ICP0 interacts with and may stabilize the cellular transcription-regulatory factor BMAL1 (Kawaguchi *et al.*, 2001). The interaction between ICP0 and elongation factor-1δ (EF-1δ) may alter translation efficiency of viral mRNAs (Kawaguchi *et al.*, 1997a). ICP0 interacts with and promotes ubiquitylation of p53 (a major oncoprotein) (Boutell & Everett, 2003); however, the relevance of this is unclear as p53 levels don't change with HSV-1 infection (Hobbs & DeLuca, 1999). ICP0 also interacts with p60, a protein of unknown function which, as mentioned previously, also interacts with ICP22 (Bruni *et al.*, 1999).

1.4.2.5.2 Phenotype of ICP0 mutants

Early studies of HSV-1 strains with both copies of ICP0 inactivated showed that although ICP0 mutants are able to replicate in tissue culture, their ability to do so is impaired compared to wild type virus (Sacks & Schaffer, 1987). Further examination by Stow and Stow (1986) revealed that this characteristic of ICP0 mutants is both MOI and cell-type dependent. For example, with a low MOI of ICP0 mutant the infectious virus yield from baby hamster kidney cells was far lower than wild type. The ICP0 mutant yield was reduced even further on other cell types such as Vero and human foetal lung cells. However, when the MOI of the ICP0 mutant was raised high enough, all three cell lines yielded similar numbers of infectious virus (Stow & Stow, 1986). On a permissive cell type for ICP0 mutants, human U2OS osteosarcoma cells, the plating efficiency of ICP0 mutants is almost indistinguishable from wild type even at low MOI (Yao & Schaffer, 1995). Because ICP0 and latency associated transcripts (LATs, described in more detail in Section 1.5) occupy the same region of the viral genome, it was difficult to determine if the phenotype was due to the ICP0 or the LAT mutation. However, LAT$^-$/ICP0$^+$ mutant viruses are not inhibited at low MOI, and cells that express ICP0, complement ICP0 mutants: indicating that mutations in LATs are not responsible for the phenotype of ICP0 mutants (Cai & Schaffer, 1992).

Recently, Everett and colleagues undertook a detailed study of the fate of ICP0 mutants upon infection of a restrictive cell type. Using single-cell-based assays they found that infection of human fibroblast cells with a low MOI of an ICP0 mutant has outcomes that vary from quiescence, to stalled infection at an in-between stage, through to productive infection (Everett *et al.*, 2004a). As was seen in earlier studies, the probability of productive infection of an ICP0 mutant was found to be MOI dependent, and appears to have a threshold. An initial infectious dose above this threshold leads to replication of the mutant virus at levels comparable to wild type. However, below that threshold, the probability of a nonproductive infection is high (Everett *et al.*, 2004a).

Everett et al found that in cells where the ICP0 mutant is unable to complete the lytic cycle, the infection stalls at a variety of stages. In these cells there is a fairly high probability of expressing the IE genes, but the expression of E genes is random and incomplete (Everett *et al.*, 2004a). These findings are consistent with results from Cai et al, who examined the effects of the ICP0 mutation on growth in Vero cells. In this study, although the levels of IE gene products from the ICP0 mutant was not significantly lower than wild type, there were drastically reduced levels of E and L gene products (Cai & Schaffer, 1992). This demonstrates that activation of IE gene expression by

ICP0 may be somewhat redundant in the presence of VP16, however ICP0 is important for up-regulation of the E and L genes (Cai & Schaffer, 1992). These results may also indicate that, especially for low MOI of infection, the viral genome of ICP0 mutants is susceptible to a cellular repression mechanism that acts on the genome after the initial burst of IE gene expression has occurred (Everett *et al.*, 2004a). Everett describes the process of repressing the viral genome as a competition between a cellular repression mechanism and viral transcription activators. ICP0 gives an advantage to the virus by promoting the degradation of cellular proteins that may be involved in repressing the viral genome (Everett, 2000b).

Considering cellular repression of the viral genome, there are a number of possibilities that could explain the MOI dependency of the ICP0 mutant phenotype. One popular explanation is that, with high viral genome load, the cellular repression mechanism is titrated out. Another explanation put forth by Everett and colleagues is that the repression of the viral genome may be random and incomplete, thereby affecting some areas but not others. In this scenario, a few of the essential products expressed from each of a number of different genomes are able to lead to a full complement of necessary proteins to allow a productive infection (Everett *et al.*, 2004a). However, additional features of the infected cell are important in determining the outcome of an ICP0 mutant infection, as there are times when productive infection occurs with very low MOI.

1.4.3 E gene expression and DNA replication

Maximum synthesis of the E gene products occurs 5-7 hrs post-infection (Honess & Roizman, 1974). As described in previous sections, expression of E genes requires ICP4 and is promoted by ICP27, and ICP0. There are two groups of E genes, roughly divided based on the timing of their expression. The first group is expressed just shortly after IE genes: for example ICP8. The second group appears after more of a delay, for example thymidine kinase (TK) (Honess & Roizman, 1974). In general, the early gene proteins are involved in DNA replication and nucleotide metabolism.

Viral DNA replication occurs in specialized nuclear structures called replication compartments (Plummer *et al.*, 1969). Upon entry into the nucleus, the parental genome localizes to the periphery of ND10 structures (Ishov & Maul, 1996). Several E proteins assemble onto the parental genome (Kristie & Roizman, 1984) forming prereplicative sites. With progression of DNA synthesis globular replication compartments form (Quinlan *et al.*, 1984). Initially the replication compartments are near the nuclear membrane, but as viral DNA replication progresses, their size increases until the entire nucleus is affected (Randall & Dinwoodie, 1986).

Replication begins from one of three origins of DNA replication. OriL is a 144bp palindrome located in the U_L segment. OriS is a 45bp palindrome located in the repeat region flanking the short segment and so is present in two copies (Taylor *et al.*, 2002). There are seven E genes that are essential for viral DNA replication (Challberg, 1986). The replication origins contain binding sites for U_L9, the viral origin binding protein. U_L9 bends DNA, causes a single-stranded (ss)-stem loop structure and recruits ICP8 (Elias *et al.*, 1986). ICP8 (U_L29), is a ssDNA binding protein (Conley *et al.*, 1981) that stimulates the helicase activity of U_L9 (Boehmer *et al.*, 1993). A heterotrimer composed of $U_L5/U_L8/U_L52$ is the helicase-primase complex and has 5' to 3' activity (Crute *et al.*, 1988). U_L30/U_L42 is the DNA polymerase with 3' to 5' exonuclease activity for proofreading: U_L42 increases processivity (Purifoy *et al.*, 1977). Cellular enzymes such as DNA ligases and topoisomerases are also likely recruited for viral DNA replication (Taylor *et al.*, 2002). Other E gene products that are nonessential in cell culture are often required for replication in non-dividing cells. Examples of these are products involved in nucleotide metabolism such as thymidine kinase (Kit & Dubbs, 1965), ribonucleotide reductase (Bacchetti *et al.*, 1986), and uracil N-glycosylase (Caradonna *et al.*, 1987).

The model for HSV-1 replication that, until recently, was widely accepted assumes that the linear HSV genome circularizes upon entry into the nucleus (by an unknown mechanism) and undergoes theta form replication. At some point, in this model, replication switches from theta to a rolling circle mode analogous to bacteriophage lambda replication. (Roizman & Knipe, 2001). However, recent evidence found by Jackson and DeLuca indicates that circular DNA is not a prerequisite for HSV-1 replication. Instead, circularization of the genome was found to occur only in the absence of ICP0 and may be an indicator of a repressed or latent genome, rather than a transcriptionally active genome (Jackson & DeLuca, 2003). In addition to this, the lambda model predicts the formation of long, linear concatemers, and does not account for the complex, branched structures found for HSV replication intermediates (Severini *et al.*, 1996). Currently, replication mechanisms that do not include circularization of the viral genome, such as a recombination-dependent mechanism that is analogous to bacteriophage T7 replication (Nimonkar & Boehmer, 2003), are being studied for relevance to HSV replication.

1.4.4 L gene expression

L gene expression occurs after the initiation of viral DNA synthesis, and in general, is at its peak 7-9 hrs post-infection (Honess & Roizman, 1974). The products of L genes are often structural proteins or are involved in the assembly of virions or modification of cellular membranes. L gene transcription occurs in replication compartments (Phelan *et al.*, 1997) and, as described in earlier sections, requires ICP4, ICP27, and ICP22 (for a subset). ICP8, in addition to its role in DNA replication, is also necessary for transcription of late genes (Gao & Knipe, 1991). The ssDNA binding protein ICP8 may act by making L promoters accessible by viral genome rearrangement or by promoting transcription from progeny genomes (McNamee *et al.*, 2000).

There are two classes of late genes. Transcription of some late genes, referred to as leaky-late genes, occurs before DNA replication. Examples of these are U_L27 and U_S6, which encode the viral glycoproteins gB and gD respectively (Roizman & Knipe, 2001). Although expression of these genes does not depend upon replication of viral DNA, it is enhanced following DNA replication. In contrast, true late gene transcription occurs only after the initiation of DNA replication. Examples of true late genes are U_L44, encoding the viral glycoprotein gC, and U_S11, which encodes an RNA binding protein (Roizman & Knipe, 2001).

1.4.5 Virion assembly and egress

Proteins involved in capsid formation localize in the nucleus and the assembly of capsids, which does not require cellular factors (Newcomb *et al.*, 1994), occurs in viral replication compartments (de Bruyn Kops *et al.*, 1998). Empty shells composed of 4 late gene products assemble on an internal scaffolding structure formed by the L gene products VP21, preVP22a and VP24 (Homa & Brown, 1997). As described earlier, the major structural protein of the outer shell is VP5, with the outer tips of the hexons formed by VP26 (Steven & Spear, 1996). The capsomeres are connected by triplexes composed of one molecule of VP19C and two molecules of VP23 (Newcomb *et al.*, 1993). Once assembled, the capsids take up DNA until either "head-full" or the recognition of certain sequences signals that the incorporation of the entire genome is complete (Roizman & Knipe, 2001). The insertion of viral DNA into the capsid displaces the internal scaffolding structure. Viral DNA replication results in complex, branched, concatemeric DNA (Severini *et al.*, 1996) that is cleaved into unit-length genomes during encapsidation. Cleavage occurs at specific packaging elements, *pac*1 and *pac*2, that are located at the ends of the viral genome (Deiss *et al.*, 1986, Mocarski & Roizman, 1982, Varmuza & Smiley, 1985) in a process that requires six viral genes: U_L6, U_L15, U_L17, U_L28, U_L32 and U_L33 (Roizman & Knipe, 2001).

Acquisition of the envelope and exit of the virion from the host cell likely occurs through a process that involves envelopement, deenvelopment, and reenvevelopment steps (Farnsworth *et al.*, 2003, Skepper *et al.*, 2001). Once DNA has been taken up by the capsid, the nucleocapsid can bud through the inner nuclear membrane into the perinuclear space, acquiring an envelope (Mettenleiter, 2002). The envelope is lost when it fuses to the outer nuclear membrane; releasing the free nucleocapsid into the cytoplasm where a tegument layer is acquired (Mettenleiter, 2002). The tegumented nucleocapsid binds to cytoplasmic membranes that are enriched with viral glycoproteins (Farnsworth *et al.*, 2003, Johnson & Huber, 2002). Virions acquire an envelope by budding into post-ER cytoplasmic compartments (Skepper *et al.*, 2001, Whiteley *et al.*, 1999) and are transported to the cell surface.

1.5 HSV-1 Latent Infection

Following replication at the site of infection, progeny virus infects sensory neurons and replicates. Within a few days, replication is replaced by a latent state (Stevens & Cook, 1971). During latency, the viral genome is retained in a non-replicating state in the neuronal nucleus where it is wrapped in a regular nucleosomal structure (Deshmane & Fraser, 1989) and has an endless or circular conformation (Mellerick & Fraser, 1987, Rock & Fraser, 1983, Rock & Fraser, 1985). The copy number of viral DNA/neuron varies from one to thousands (Sawtell, 1997). None of the lytic genes are expressed during latency and only latency associated transcripts (LATs) accumulate to high levels. Even neuron specific promoters are repressed in context of viral genome (Glorioso *et al*., 1995, Lachmann *et al*., 1996). The structural basis for the repression of gene expression during latency is unknown, but it is assumed that the cell treats the viral genome as set of genes to be silenced (Preston, 2000). Recent experiments indicate that a major regulatory determinant of gene expression during HSV latency may be histone composition (Kubat *et al*., 2004). Using chromatin immunoprecipitation assays of latent DNA isolated from mouse dorsal root ganglia, Kubat and colleagues demonstrated that a portion of the LAT region is associated with histone H3 acetylated at lysines 9 and 14: characteristic of euchromatic or nonrepressed DNA. In contrast, chromatin associated with HSV-1 DNA Polymerase (in the U_L segment) was not enriched in H3 acetylated at lysines 9 and 14, consistent with an inactive region of DNA (Kubat *et al*., 2004).

The expression of LATs is a hallmark of HSV latency. The LAT promoter functions efficiently in neurons (Kosz-Vnenchak *et al*., 1990, Spivack & Fraser, 1988), leading to the production of an 8.3 kb transcript that is antisense to ICP0 mRNA (Roizman & Knipe, 2001). Only low levels of the 8.3 kb transcript accumulate; the much more abundant 2.0 and 1.5 kb introns (Krause *et al*., 1988, Rock *et al*., 1987, Spivack & Fraser, 1987) have an unusual lariat structure that contributes to their stability (Farrell *et al*., 1991, Wu *et al*., 1996). Sixty-seven to ninety-five percent of infected neurons do not express detectable levels of LATs (Maggioncalda *et al*., 1996, Ramakrishnan *et al*., 1994, Sawtell, 1997), indicating that LATs are not absolutely essential for any aspects of latency. The function of LATs is controversial. Claims that LATs prevent the expression of ICP0 (Chen *et al*., 1997, Garber *et al*., 1997), enable reactivation, or maintain latency have all been disputed (Roizman & Knipe, 2001). LATs may prevent apoptosis (Perng *et al*., 2000) by influencing the accumulation of transcripts encoding the antiapoptotic protein Bclx(L) (Peng *et al.*, 2003). In addition to this, LATs may inhibit caspase-8 and caspase-9 induced apoptosis (Henderson *et al.*, 2002, Jin *et al.*, 2003).

Establishment of latency does not require a specific viral function or protein (Preston, 2000, Wagner & Bloom, 1997). A widely accepted hypothesis is that latency occurs by default in the absence of IE gene expression. A factor that may be involved in the inhibition of IE gene expression is that host cell proteins important for IE transcription are unavailable in the nucleus of neurons (Kristie *et al.*, 1999, Valyi-Nagy *et al.*, 1991). For example, in sensory neurons HCF is sequestered exclusively in the cytoplasm (Kristie *et al.*, 1999) and is translocated to the nucleus only under certain conditions such as explantation of ganglia, leading to reactivation (Kristie *et al.*, 1999). Other cell types have nuclear or general distribution of HCF (Kristie *et al.*, 1995, La Boissiere *et al.*, 1999). Recent evidence suggests that a post-IE block may be responsible for the establishment of the latent state. The nervous system is enriched for NFB42, an F-box protein that is part of ubiquitin-ligase complexes responsible for the degradation of phosphorylated proteins in a proteasomal dependent manner (Feldman *et al.*, 1997, Skowyra *et al.*, 1997). The HSV origin binding protein, U_L9, (essential for DNA replication) is polyubiquitinated in vivo and binds NFB42 (Eom & Lehman, 2002). There is a significant decrease of U_L9 when NFB42 and U_L9 are co-expressed in 293T cells and MG132 treatment restores U_L9 levels (Eom & Lehman, 2003). The model proposed by the authors is that in neurons U_L9 is phosphorylated, recognized by NFB42 and degraded. The lack of U_L9 inhibits initiation of viral DNA replication and may lead to the establishment of latency (Eom & Lehman, 2003).

Attempts to develop a tissue culture model of latency in fibroblast cells led to the discovery that a non-replicating or quiescent state could be established if IE gene expression was prevented upon entry of the virus into the cell (Roizman & Knipe, 2001). However, in this tissue culture model it appears that the quiescent genome is globally repressed and requires major changes before expression is possible (Preston, 2000). In contrast, the latent genome in animal models seems to be less severely repressed, is more susceptible to reactivation stimuli, and key promoters, such as the LATs, are responsive to neuronal signals (Preston, 2000). Because of this, HSV quiescence in fibroblast cells is not considered to be an accurate model of latency and is instead studied to gain an understanding of cellular antiviral responses. Quiescence will be discussed in more detail in section 1.6. Cultured neurons either infected with low MOI or in conjunction with DNA synthesis inhibitors such as acycloguanosine are also used for a tissue culture model for latency (Wilcox & Johnson, 1988, Wilcox & Johnson, 1987). With this system replication is prevented, and the 2 kb LAT species is expressed (Smith *et al.*, 1994); however, there are concerns that this approach is artificial as well.

Periodic reactivation of latent HSV occurs in only a fraction of infected neurons (Roizman & Knipe, 2001). There appears to be a correlation between reactivation and the amount of viral DNA/neuron and the number of infected neurons (Lekstrom-Himes *et al.*, 1998, Sawtell, 1998, Sawtell *et al.*, 1998). The data suggests that a higher viral DNA copy number leads to a predisposition towards reactivation (Sawtell *et al.*, 1998). Stressful treatments of mice and other animals induces reactivation in vivo (Kwon *et al.*, 1981, Sawtell & Thompson, 1992). Also, providing ICP0, ICP4 or VP16 in trans induces reactivation of latently infected trigeminal ganglion (Halford *et al.*, 2001). Reactivation from latency could involve HCF, which is part of the E transcription activating complex (Kristie *et al.*, 1999). Assuming the latent genome is accessible to transactivators, stimuli for reactivation of the latent genome causing HCF to be transported from cytoplasm to the nucleus may lead to activation of IE transcription (Kristie *et al.*, 1999). Any specific viral protein is not essential for reactivation from latency but, in animal models, there is poor reactivation of ICP0 mutants from latency in neurons (Cai *et al.*, 1993, Leib *et al.*, 1989). Small amounts of ICP0 initially expressed may work to reverse repression of the rest of the genome (Everett, 2000b). However, there is insufficient data to determine if the importance of ICP0 in reactivation from latency is to promote viral gene expression or to somehow convert the viral genome from a silent state to an active state (Preston, 2000). Also, the poor reactivation of ICP0 mutants could be due to inefficient replication at nerve endings, resulting in a low viral DNA copy number.

1.6 HSV-1 Quiescence

Infecting cells with viruses that are unable to express the IE genes results in an abortive infection where the viral genome is retained by the cell in a quiescent form (Roizman & Knipe, 2001). A number of viruses that are deficient in IE gene expression have been developed. For example, the VP16/ICP0 mutant KM110 (Mossman & Smiley, 1999), the VP16/ICP0/ICP4 mutant in1820K (Preston & Nicholl, 1997), and the ICP0/ICP4/ICP22/ICP27/ICP47 mutant d109 (Samaniego *et al.*, 1998). Even at high MOI these viruses are nontoxic in Vero and HEL cells (Mossman & Smiley, 1999, Preston & Nicholl, 1997, Samaniego *et al.*, 1998) and instead establish a persistent infection.

During quiescence there is no viral gene expression, including expression of LATs (Harris & Preston, 1991, Preston & Nicholl, 1997, Samaniego *et al.*, 1998). Initially following infection the viral promoters are responsive to transactivators such as VP16, hexamethylene bisacetamide (HMBA), and the human cytomegalovirus (HCMV) homolog of VP16 (Harris & Preston, 1991, Preston & Nicholl, 1997, Samaniego *et al.*, 1998). However, once the genome has been converted to a repressed state it is very resistant to reactivation.

After 24 hrs the promoters become unresponsive to transcriptional activators such as VP16, HMBA, and ICP4 (Harris & Preston, 1991, Preston & Nicholl, 1997, Samaniego *et al.*, 1998, Stow & Stow, 1989). Even stimuli such as host cell division does not lead to reactivation (Jamieson *et al.*, 1995).

The repression of the quiescent genome works on all HSV-1 and heterologous promoters that have been tested to date and is therefore not likely to be sequence specific (Preston, 2000). For example, apart from the TATA box, there are no common sequence motifs for the four HSV IE and the HCMV IE promoters, but these are all shut off (Preston & Nicholl, 1997, Samaniego *et al.*, 1998). Even cellular promoters such as for beta-actin are silent in the context of the quiescent genome (C.M Preston, unpublished observations). Similarly, the ICP0 promoter, when present on both the cellular and the HSV-1 genomes, is active from the cellular genome but not from the quiescent virus (Preston & Nicholl, 1997).

The structure of the quiescent genome, and therefore the mechanism of its repression, is still largely unknown. The quiescent genome does not have a regular nucleosomal organization (Jamieson *et al.*, 1995) and is a nonlinear molecule (Harris & Preston, 1991, Jamieson *et al.*, 1995, Preston & Nicholl, 1997, Samaniego *et al.*, 1998). Recently, it was shown that a fraction of d109 genomes circularize upon infection (Jackson & DeLuca, 2003). The repression mechanism of quiescent genomes may involve heterochromatin. Heterochromatin is a condensed form of DNA found at chromosomal regions that are transcriptionally silent such as centromeres and telomeres (Elgin, 1996). Even genes close to heterochromatin can be silenced: a phenomenom discovered in Drosophila and called position effect variegation (Eissenberg *et al.*, 1990, Eissenberg *et al.*, 1992). In Drosophila, the heterochromatin protein HP-1 promotes position-effect variegation (Eissenberg *et al.*, 1992). HP-1 is a component of centromeres and ND10 (Everett *et al.*, 1999b, Lehming *et al.*, 1998, Seeler *et al.*, 1998) that forms a complex with another ND10 component Sp100 (Lehming *et al.*, 1998, Seeler *et al.*, 1998). ICP0 may disrupt ND10 structures in order to prevent repression of the viral genome. It is speculated that the localization to and disruption of ND10 by ICP0 prevents HP-1 from interacting with heterochromatin (Preston, 2000).

The repression of the quiescent genome lasts for long periods; however, the persisting genomes are still functional and can be induced to reactivate days or even weeks later (Mossman & Smiley, 1999, Preston & Nicholl, 1997, Samaniego *et al.*, 1998). Provision of ICP0 from another source is sufficient to reverse repression and lead to reactivation of the quiescent genomes (Everett *et al.*, 1998b, Harris & Preston, 1991, Stow & Stow, 1989). ICP0, its functional homologs from other herpes viruses, and the histone deacetylase (HDAC) inhibitor trichostatin A (TSA) are the only known agents that can reverse repression (Everett *et al.*, 1998b, Harris & Preston, 1991, Hobbs & DeLuca, 1999, Preston & Nicholl, 1997, Samaniego *et al.*, 1998, Stow & Stow, 1989). Reactivation of quiescent viral genomes by ICP0 depends on proteasome activity and is prevented by the proteasome inhibitor MG132 (Everett *et al.*, 1998a).

1.7 Intracellular Antiviral Responses

The ability of cells to detect and respond to viral invasion is critical for preventing the spread of viruses. Interferons (IFNs) play a major role in this response by stimulating the expression of cellular factors that make the cell inhospitable for viral replication and warn neighbouring cells to do the same. The products of interferon stimulated genes (ISGs) are the important effectors in preventing viral replication. These can be induced by both IFN-dependent and IFN-independent pathways. Like all successful viruses, HSV-1 has evolved mechanisms to combat the effects of the antiviral response it induces upon infection of cells. ICP0 in particular has a major role in overcoming the antiviral response and ensuring that the environment of the cell is conducive to viral growth.

1.7.1 Interferon response

The immunomodulatory and antiproliferative actions of interferons are involved in diverse functions such as protection against tumour cells and pathogens such as viruses, bacteria, and parasites (Stark *et al.*, 1998). IFNs were first discovered in 1957 by Isaacs and Lindenmann who found that a protein in the medium harvested from cells infected with a heat-inactivated virus could "interfere" with subsequent infection of cells by a live virus (Isaacs & Lindenmann, 1957). Since then their importance as an early line of defense against viral infection has been made very clear. For example, IFN receptor knockout mice are extremely sensitive to both vaccinia and lymphocytic choriomeningitis virus infections compared to wild type mice (van den Broek *et al.*, 1995).

The two basic groups of IFNs are referred to as Type I, or viral IFNs, (monomers) and Type II, or immune IFNs, (dimers). The major Type I IFNs, IFN-α and IFN-β, are produced mainly by leukocytes and fibroblasts, respectively, and bind to the same IFN-α/β receptor (Andrea *et al.*, 2002, Samuel, 2001). The major Type II interferon, IFN-γ, is produced mainly by lymphocytes and NK cells, and binds to the IFN-γ receptor (Andrea *et al.*, 2002, Samuel, 2001).

The signaling pathways of Type I and Type II IFNs are related. In both pathways, binding of IFN to the receptor initiates a cascade of phosphorylations beginning with the receptor-associated Janus kinases (JAKs), and leading to the phosphorylation and dimerization of the signal transducers and activators of transcription (STATs) (Stark *et al.*, 1998). The activated STAT dimers are released from the receptors and transported to the nucleus where they induce the transcription of ISGs by binding to either gamma-activated sequence (GAS) or IFN-stimulated regulatory elements (ISREs) (Stark *et al.*, 1998).

Specificity for either ISRE or GAS elements is influenced by factors such as the combination of STATS in the dimer, and the interaction of the dimer with a variety of other transcription factors (Stark *et al.*, 1998). Activated STAT1 homodimers form in response to IFN-γ signaling and induce ISGs by binding to specific GAS elements (Darnell *et al.*, 1994, Schindler & Darnell, 1995). The major transcription factor formed in response to IFN-α/β signaling is a trimer called ISGF3, which is composed of a STAT1-2 heterodimer bound to p48 (a member of the interferon regulatory factor family) (Stark *et al.*, 1998). ISGF3 binds to specific ISREs sequences and drives the expression of most ISGs (Bluyssen *et al.*, 1995, John *et al.*, 1991). Also following IFN-α/β signaling, STAT1 homodimers and STAT1-2 heterodimers can form independently of p48. These bind GAS elements and can drive the expression of other ISGs (Haque & Williams, 1994, Li *et al.*, 1996). Possibly due to secondary modifications, the STAT1 homodimer formed in response to IFN-α/β signaling does not induce the same ISGs as does the STAT1 homodimer formed in response to IFN-γ (Haque & Williams, 1994). These examples illustrate how a common pathway can lead to the expression of different combinations of ISGs that are tailored to respond effectively to a diversity of threats.

1.7.2 IFN-stimulated genes

The human genome encodes between 600 and 2,000 interferon stimulated genes (deVeer *et al.*, 2001); however, an antiviral state capable of inhibiting virus replication appears to require the induction of only a small group of ISGs. ISGs cause an antiviral state by targeting many steps of virus replication. For example, activation of PKR leads to the inhibition of translation of both viral and cellular mRNA. (Clemens & Elia, 1997, Samuel, 1993). DsRNA triggers the activation and autophosphorylation of PKR, which can then associate with ribosomes and phosphorylate the eukaryotic initiation factor eIF-2α (Samuel, 1979). Phosphorylation of eIF–2α impairs the eIF-2B-catalyzed guaninie nucleotide exchange reaction, thereby inhibiting the translation of mRNA (Clemens & Elia, 1997, Samuel, 1993). Two ISGs, 2'5'-Oligoadenylate synthetase (OAS) and RNaseL, lead to the degradation of RNA (Samuel, 2001). DsRNA activates OAS which can then catalyze the formation of oligoadenylates with a 2'5'-phosphodiester bond linkage called 2'5'-Oligo Adenylic Acid (2-5A) (Kerr & Brown, 1978). 2-5A oligomers bind to the latent endoribonuclease RNase L, converting the inactive monomer to the active homodimer that degrades RNA (Dong & Silverman, 1995). The IFN inducible RNA-specific adenosine deaminase ADAR1 converts adenosine (A) to inosine (I) (Patterson *et al.*, 1995). This leads to RNA editing because I is recognized as guanine (G) instead of A by ribosomes and polymerases (Samuel, 2001). Mx family proteins such as MxA and Mx1 are GTPases from the superfamily of dynamin-like GTPases (Van der Blick, 1999). In the absence of any other ISG products, Mx can prevent the replication of enveloped ssRNA viruses (Arnheiter *et al.*, 1996, Regad & Chelbi-Alix, 2001). The GTPase interacts with viral nucleocapsids, interfering with their nuclear import and thereby preventing transcription (Kochs & Haller, 1999). ISG 56K binds to the p48 subunit of the eukaryotic translation initiation factor 3 (eIF-3), inhibiting its function (Guo *et al.*, 2000).

IFN treatment results in an increase in the size and number of ND10 structures (Taylor *et al.*, 2000). At least 6 ND10 proteins are upregulated by IFN including: PML, Sp100, Sp110, Sp140, ISG20 and PA28 (Regad & Chelbi-Alix, 2001). Overexpression of PML inhibits VSV and influenza virus (Chelbi-Alix *et al.*, 1998), however the mechanism for this is unknown. Chee and colleagues found that HSV ICP0 mutants are very sensitive to IFN-α and -γ in

PML$^{+/+}$ murine cells, while wild type virus is not, and that neither virus is affected by IFNs in PML$^{-/-}$ cells (Chee *et al.*, 2003). These results suggest that PML mediates an anti-viral state induced by IFN; therefore, the virus targets ND10 and disrupts PML to avoid this inhibition (Chee *et al.*, 2003). However, HSV-1 replication appears to be unaffected by the overexpression of PML despite the fact that ND10 structure remain intact (Lopez *et al.*,.2002).

1.7.3 IFN-independent response

A number of alternate signaling pathways can lead to the induction of ISGs in the absence of interferon. For example: the binding of HCMV glycoprotein B to human fibroblasts, (Boyle *et al.*, 1999) and the presence of dsRNA (Bandyopadhyay *et al.*, 1995, Tiwari *et al.*, 1987). Recently, it has been discovered that infection of cells by HSV-1 can lead to an antiviral state in an IFN-independent manner. This aspect of HSV-1 infection was hidden by the ability of wild-type virus to prevent the induction of ISGs when infection is allowed to proceed normally (Mossman *et al.*, 2001, Nicholl *et al.*, 2000).

Experiments where HSV-1 gene expression was prevented following infection revealed the induction of ISGs (Eidson *et al.*, 2002, Mossman *et al.*, 2001, Nicholl *et al.*, 2000). Microarray analysis of cells infected with KM110, a VP16/ICP0 mutant severely defective in gene expression (Mossman & Smiley, 1999), or UV-inactivated KOS showed the induction of a subset of genes very similar to what was induced by IFN (Mossman *et al.*, 2001). Examples of these genes include MXI, OAS, and PML (Mossman *et al.*, 2001) which, as described previously, act to limit virus replication. Preston and colleagues found that ISG 54, IFI 56, ISG 15, 9-27 and MxA were induced upon infection of cells with HSV-1 under conditions where protein synthesis was prevented with cycloheximide (Nicholl *et al.*, 2000). Similarly, microarray analysis of cells infected with another HSV-1 mutant incapable of gene expression, d109 (mutant in all 5 IE genes (Samaniego *et al.*, 1998)), led to the expression of many of the same ISGs as was seen by Mossman et al and Nicholl et al (Eidson *et al.*, 2002).

The antiviral response induced by HSV-1 is able to prevent plaque formation of a diverse set of RNA and DNA viruses. For example, HSV-1, vesicular stomatitis virus (VSV), and vaccinia were unable to form plaques on plates previously infected with the HSV-1 mutant KM110 (Mossman *et al.*, 2001). Similarly, high MOI of the HSV-1 mutant d109 also lead to inhibition of wild type virus plaquing (Eidson *et al.*, 2002).

Independent lines of evidence strongly support that the induction of ISGs by HSV-1 occurs in the absence of IFN. Medium from infected cells did not induce expression of ISGs (Nicholl *et al.*, 2000) or lead to the inhibition of plaque formation (Mossman *et al.*, 2001). Also, the JAK/STAT components (TYK2, STAT1 and JAK1) that are important mediators of the IFN-α response were shown to be unnecessary for the induction of ISG54-specific RNA by HSV-1 (Nicholl *et al.*, 2000).

Entry of the HSV-1 virion is required for the antiviral state to be triggered in human fibroblasts (Mossman *et al.*, 2001, Preston *et al.*, 2001). Comparisons of the ability of viral envelope glycoprotein mutants in gD and gB showed that these viruses which are unable to enter cells do not induce ISG 56K RNA, a transcript strongly induced by KM110 infection (Mossman *et al.*, 2001). Similarly, gD and gH glycoprotein deficient viruses do not induce ISG 54-specific RNA (Preston *et al.*, 2001).

Further research done by Mossman and colleagues broadened the understanding of this occurrence by showing enveloped viruses in general, not just HSV-1, trigger an IFN-independent antiviral response following infection. The ability of a diverse set of UV-inactivated viruses to induce ISGs was compared with HSV-1 and vaccinia virus representing enveloped dsDNA viruses, VSV, New-Castle disease virus (NDV), and simian virus (SV) representing enveloped –ve ssRNA viruses, and Adenovirus representing the noneveloped, dsDNA viruses. Of these viruses, only Adenovirus did not lead to the accumulation of ISG 56K or prevent plaque formation, indicating that the induction of the antiviral response in human and mouse fibroblasts requires an envelope (Collins *et al.*, 2004).

Important components involved in the induction of ISGs in response to entry of enveloped viruses have recently been discovered. Experiments done by Mossman and colleagues using primary mouse embryo fibroblasts deficient for various interferon response factors (IRFs) showed that the induction of ISGs in response to UV-inactivated HSV-1, VSV, VV, NDV, and SV requires IRF-3, but not IRF-1, IRF-7, or IRF-9 (Collins *et al.*, 2004). Further evidence for the involvement of IRF-3 comes from experiments done by Preston et al, who found that HSV-1 infection, in the presence of cycloheximide, induces a DNA-binding complex capable of recognizing an interferon-responsive sequence motif. The complex capable of binding ISREs includes the CREB-binding protein and IRF-3 (Preston *et al.*, 2001). Similar to the expression of ISGs, there was a severe decrease in the amounts of this complex under conditions of normal HSV-1 replication (Preston *et al.*, 2001).

Following stimuli such as virus infection or dsRNA, IRF-3 is activated by phosphorylation and dimerizes. Activated IRF-3 is translocated to the nucleus where it induces ISGs (Peters *et al.*, 2002, Toshchakov *et al.*, 2002). Recently, two groups have shown that activation of IRF-3 in response to viruses or dsRNA involves IKB kinase homologs; IKB kinase-ε (IKKε) and TANK-binding kinase-1 (TBK1) (Fitzgerald *et al.*, 2003, Sharma *et al.*, 2003). Fitzgerald and colleagues also suggest the involvement of Toll-like receptor 3 (TLR3) and TRIF (TIR domain containing adaptor inducing IFN) for activation of IRF3 (Fitzgerald *et al.*, 2003).

ICP0 is very important in mediating resistance to IFN (Harle *et al.*, 2002, Mossman *et al.*, 2000). Mossman and colleagues found that wild type virus is relatively unaffected by the affects of IFN-α in tissue culture, while ICP0 mutants are severely inhibited (Mossman *et al.*, 2000). In addition to this, ICP0 mutants are not inhibited for replication in IFN-α/β receptor knockout mice (Leib *et al.*, 1999). Both expression of ICP0 and proteasome function are necessary to inhibit induction of ISGs following virus infection (Eidson *et al.*, 2002). ICP0 blocks IRF-3 and IRF-7 in a RING-finger dependent manner (Lin *et al.*, 2004); however, as it is still unknown how HSV-1activates the IRF-3 pathway, the exact mechanism ICP0 uses to inhibit IRF3 activity is unclear as well (Lin *et al.*, 2004). ISG induction is not blocked by the degradation of proteins known to be part of the virus induced IRF3 pathway including TBK-1, IKKε, IRF-3, and CBP (Lin *et al.*, 2004), therefore, further study is needed to determine the mechanism.

1.8 Experimental Rational

Previous experiments done by Harris et al suggest that ICP0 mutants are incapable of reactivating a quiescent genome. In these experiments fibroblast cells were infected with a low MOI HSV-2 to establish quiescence then superinfected with an ICP0 mutant (Harris *et al.*, 1989). There was no evidence of replication of the HSV-2 genome, although there was evidence that the ICP0 mutant had replicated. This result is interesting because it suggests that, although one viral genome is able to undergo transcription and replication, another, almost identical viral genome is prevented from doing the same. However, these results are ambiguous because the low MOI of HSV-2 used to establish quiescence, combined with the low MOI of ICP0 mutant used for superinfection leads to the possibility that the ICP0 mutant replicated strictly in cells that did not harbour quiescent HSV-2.

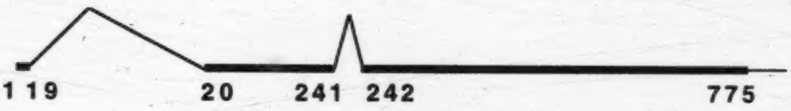
Research done by Mossman et al and Nicholl et al led to the suspicion that ICP0 mutants may be incapable of infecting cells that harbour a quiescent genome. They found that, upon infection, the virus particle induces an antiviral response in cells (Mossman *et al.*, 2001, Nicholl *et al.*, 2000). This anti-viral response leads to the induction of many of the same genes as the interferon response. However, unlike wild-type HSV-1, the quiescent mutant is incapable of disarming this response (Mossman *et al.*, 2001, Nicholl *et al.*, 2000). In addition to this, ICP0 mutants are far more sensitive to IFN-α than is wild-type HSV-1 (Mossman *et al.*, 2000). Therefore, when I began this project, it was unknown whether the presence of a replicating, ICP0 mutant HSV-1 genome is capable of reactivating a quiescent genome.

My project has been to develop an experimental system that can be used to determine if ICP0 mutants are capable of productively infecting cells that harbour a quiescent genome. To do this, I constructed fluorescent recombinant HSV-1 strains. Products resulting from the quiescent genome and the superinfecting genome can be discriminated by: colour of the fluorescent protein, presence of genomic restriction sites, and protein size. With these viruses I was able to determine the proportion of cells that harbour a quiescent genome, as well as the proportion that could be productively superinfected with the ICP0 mutant. Using a high MOI of both infecting and superinfecting viruses, I have been able to determine that an ICP0 mutant is capable of overcoming the antiviral response and replicating in cells that harbour the quiescent genome. However, superinfection with the ICP0 mutant does not lead to reactivation of the quiescent genome. The quiescent genome remains silent despite the presence of all the necessary transcription and replication machinery, indicating that it has been converted into an inaccessible state or put into an inaccessible place.

Figure 1.1: Schematic diagrams of the ICP0 transcript and functional domains.

(A) ICP0 transcript showing 2 introns (thin inverted-V lines) and 3 exons (thick lines) and the 3' non-coding region (thin line). Exon 1 encodes amino acid (a.a.) residues 1-19, exon 2 encodes a.a. residues 20-241, exon 3 encodes a.a residues 242-775. From (Boutell *et al.*, 2002). (B) Diagram of ICP0 functional domains. The clear boxes indicate domain regions. RING = RING finger zinc-binding motif. NLS = nuclear localization signal. USP7 = ubiquitin specific protease 7 binding domain. ND10 = nuclear domain 10 localization domain. Multimer = self-interaction domain (same domain as ND10). The numbers indicate a.a. residues 1-775. From (Everett, 2000b).

A.


1
19
20
241
242
775

B.

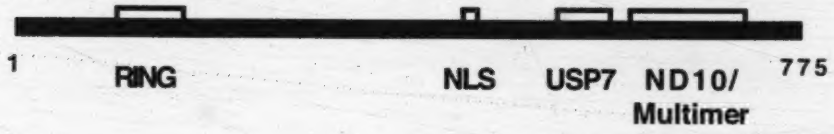
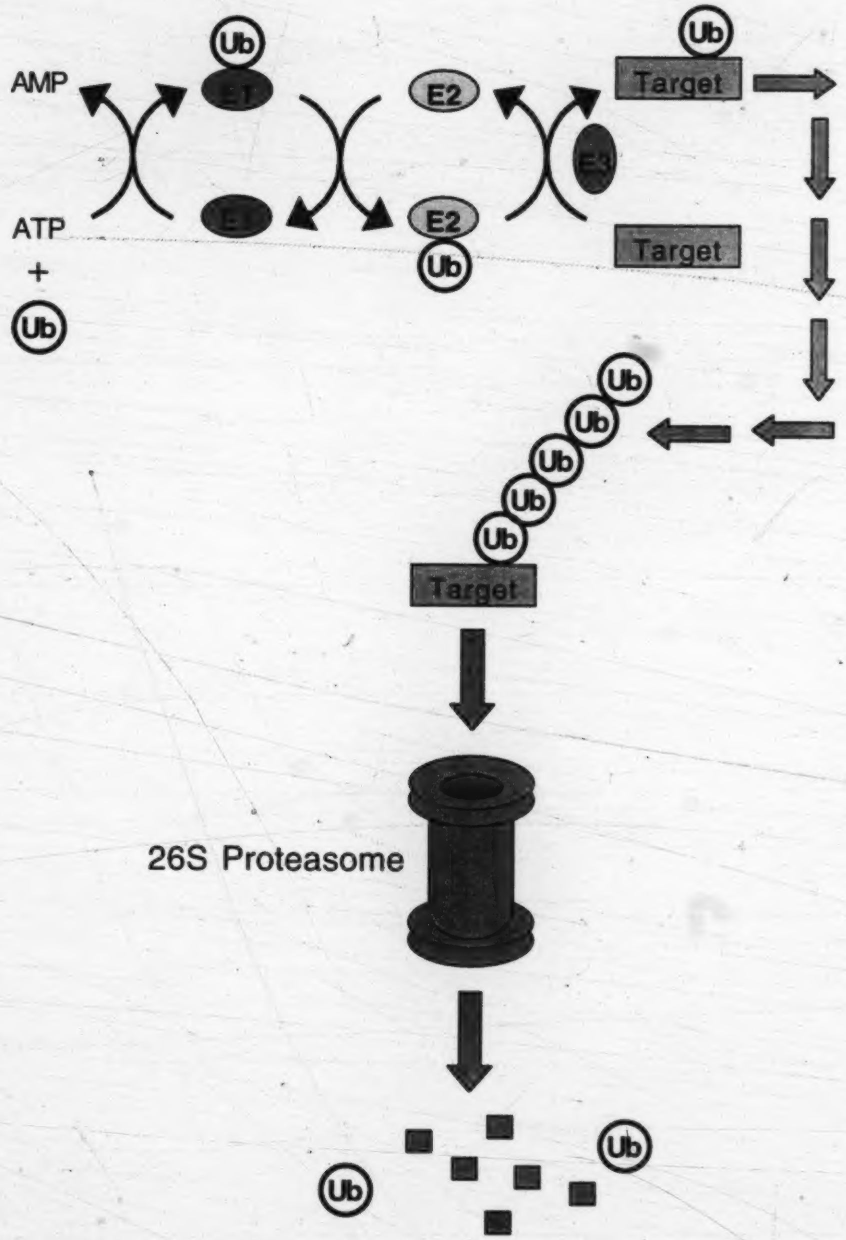

1
RING
NLS
USP7
ND10/ Multimer
775

Figure 1.2: Poly-ubiquitin signal for proteasomal degradation. Ubiquitin (Ub) is activated by the action of the E1 ubiquitin activating enzyme (E1); converting 1 molecule of ATP (adenosine triphosphate) to AMP (adenosine monophosphate). The activated ubiquitin is transferred first to the E2 conjugating enzyme (E2), then, with the help of the E3 ubiquitin ligase (E3), to the target protein (target). Poly-ubiquitination of the target protein is the signal for its degradation by the 26S proteasome. From (Sakamoto, 2002).


AMP
ATP
+
Ub
Ub
E1
E1
E2
E2
Ub
E3
Ub
Target
Target
Ub
Ub
Ub
Ub
Ub
Target
26S Proteasome
Ub
Ub

CHAPTER 2 : MATERIALS AND METHODS

2.1 Mammalian Cell Culture

Human U2OS osteosarcoma cells, Human Embryonic Lung (HEL) fibroblasts and African green monkey kidney (Vero) cells were obtained from the American Type Culture Collection. E5 and V27 (Vero derived) cells were gifts from N. A. DeLuca and S. Rice respectively. Cells were maintained in 150 cm^2 tissue culture flasks (Corning) with 25 mls of Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% (U2OS and HEL) or 5% (Vero) fetal bovine serum (FBS), 50 units/ml penicillin (P) and 5 µg/ml streptomycin (S). E5 and V27 cells were additionally supplemented with 100 µg/ml G418 (Geneticin®, GIBCO). The flasks were incubated at 37°C in a humidified atmosphere supplemented with 5% CO_2. The cells were passaged when confluent (every two to three days) by trypsinizing the cells until they were no longer attached to the flask (Trypsin, Gibco), resuspending them in DMEM/FBS, and reseeding to approximately 25% confluency.

HEL cells were passaged only 6-7 times, while U20S and Vero cells were passaged up to 45 times before thawing fresh cells. Monolayers of confluent U2OS or Vero cells were prepared by seeding 2.5×10^5 (12-well) or 5×10^5 cells (6-well) in tissue culture plates (Corning) the day before use. Monolayers of confluent HEL cells were prepared by seeding 2.5×10^5 (12-well) or 1×10^6 cells (60 mm) in tissue culture plates two days before use. The concentration of cells was determined before seeding using a hemacytometer.

2.2 Virus Strains and Growth Conditions

The following virus strains were used in this investigation:

KOS 1.1 is a wild type virus. Stocks of this virus were grown and titered on Vero cells.

n212 (Cai & Schaffer, 1989) is an ICP0 mutant virus. Both copies of the ICP0 gene bear a *Spe* I linker which contains an amber chain termination codon that results in the truncation of the ICP0 protein immediately after amino acid residue 212. Stocks of this virus were grown and titered on U2OS cells.

KM110 (Mossman & Smiley, 1999) is a VP16/ICP0 double mutant virus. The VP16 gene bears a *Nhe* I linker which contains an amber chain termination codon that results in the truncation of the VP16 protein immediately after amino acid residue 422. Both copies of the ICP0 gene of this virus are disrupted by the *Spe* I linker described above for n212. Stocks of this virus were grown and titered on U20S cells in the presence of 3mM HMBA.

d120 (DeLuca *et al.*, 1985) is an ICP4 mutant virus. Both copies of the ICP4 gene have a 4.1 kbp deletion. As only the 5' end of the ICP4 gene remains, only the amino terminus of ICP4 is encoded by d120. Stocks of this virus were grown and titered on E5 cells (DeLuca & Schaffer, 1987) (a derivative of Vero cells with an integrated copy of wild type ICP4).

d22lacZ (Long *et al.*, 1999) is an ICP22 mutant virus. All but the first six codons of the ICP22 gene are deleted. Additionally, the ICP22 gene has an in-frame insertion of the *E. coli* lacZ ORF. The resulting protein contains the first six amino acid residues of ICP22 fused to β-galactosidase. Stocks of this virus were grown and titered on Vero cells.

d27-1 (Rice & Knipe, 1990) is an ICP27 mutant virus. The ICP27 gene of the parental d27-lacZ1 virus has a 1.6 kbp deletion. This deletion prevents the production of any ICP27 related polypeptides. Stocks of this virus were grown and titered on V27 cells (Rice & Knipe, 1990) (a derivative of Vero cells with an integrated copy of wild type ICP27).

2.3 Amplification of Virus Stocks

Confluent monolayers of a permissive cell line grown in 150 cm^2 flasks were infected with an MOI of 0.1-0.5 of virus in 5 mls DMEM for 1hr at 37°C, with rocking every 15 min. The innoculum was replaced with medium 199 + P/S (Gibco) supplemented with 10% FBS. Infected cells were incubated at 34 or 37°C in a humidified atmosphere supplemented with 5% CO_2. After the majority of the cells exhibited total cytopathic effect (CPE) (3-5 days post-infection), they were pelleted by centrifugation at 2800 rpm for 10 min at 4°C in a Beckman G5-R swinging bucket centrifuge. The cell pellet was resuspended in 1 ml DMEM/flask then subjected to three rounds of freezing for 15 min at –80°C and thawing at 37°C. The cells were disrupted by three sonication cycles of 20 seconds each with a 550 Sonic Dismembrator (Fisher Scientific) at setting 7. Cell debris was separated by centrifugation at 3000 rpm for 10 min in a swinging bucket centrifuge at 4°C and the supernatant was kept as part of the final viral stock. The pellet was resupsended in 0.2 ml DMEM/flask, and the sonication and centrifugation steps were repeated. The two supernatants were combined and stored in cryovials (Nalgene) at –80°C.

2.4 Virus Titration

Virus stocks were titrated using ten-fold dilutions ranging from 10^{-2} to 10^{-7} prepared in DMEM. 0.25 ml of each dilution was used to infect each well of confluent monolayers of permissive cells grown in 12-well plates (Corning). The infection was allowed to proceed for 1 hour at 37°C, with rocking every 15 minutes. The innoculum was replaced with 1 ml of medium 199 + P/S supplemented with 10% FBS and 1% human immune serum (ICN). After plaques developed they were counted either by using a light microscope with the aid of a grid drawn on the bottom of the culture plate, or the monolayer was fixed with methanol for 10 min, then stained with 10% Giemsa stain (Sigma) for at least 1 hr.

2.5 Virus Infection

The number of cells in a confluent monolayer of cells grown on a 12-well or 60 mm plate was determined using a hemacytometer. The cells were infected with either 250 µl (12-well) or 1ml (60 mm) of virus diluted to the desired MOI in DMEM. The infection was allowed to proceed for 1 hr at 37°C, with rocking every 15. The infecting medium was replaced with 1 ml (12-well) or 5 mls (60 mm) of 10% DMEM. The plates were incubated at 37ºC in a 5% CO_2 humidified atmosphere for the indicated amount of time before being harvested for analysis.

2.6 Acid Glycine Wash

2 hrs post-infection, the growth media from monolayers of HEL cells grown in 12 well plates was aspirated. The cells were then incubated with 1 ml Acid Glycine wash (8 g/L NaCl, 1.8 g/L KCl, 0.1 g/L $MgCl_2{\bullet}6H_2O$, 0.1 g/L $CaCl_2{\bullet}6H_2O$, 7.5 g/L glycine, pH 3) for 30 seconds. After two washes with 1 ml Phosphate Buffered Saline (PBS: 10 mg/ml NaCl, 0.25 mg/ml KCl, 1.8 mg/ml Na_2HPO_4, 0.3 mg/ml KH_2PO_4, pH 7.5), regular growth media was added.

2.7 Western Analysis

Monolayers of HEL or U2OS cells grown on 12-well plates were washed with 1 ml PBS, then resuspended in 100 µl SDS PAGE lysis buffer (10mM Tris-HCl, 2% SDS, 0.005% bromophenol blue, 25% glycerol, 0.031g/ml DTT, pH 6.8). The samples were boiled at 100°C for 5 min before loading 5 µl onto a

12% acrylamide SDS PAGE gel along with 10 µl pre-stained SDS-PAGE standards, Low Range (BIO-RAD). The gel was electrophoresed for 1-2 hrs at 80 V in 1 X SDS PAGE buffer. Protein was transferred to a nitrocellulose membrane (Hybond ECL, Ambersham Pharmacia) using a wet protein transfer apparatus (Bio-Rad Trans-blot cell) at 1.8 A for 3 hrs. Following the transfer, the membrane was incubated in 10 % skim milk TBS-Tween (25 mM Tris, pH 8, 150 mM NaCl, 0.1 % Tween-20) overnight at 4°C.

The following dilutions of primary Ab were used in this investigation: monoclonal anti-VP16 (LP1) (McLean *et al.*, 1982) 1:16 000; monoclonal anti-U_S11 (Roller & Roizman, 1992) 1:10 000; and monoclonal anti β-actin (Sigma Aldrich) 1:5 000. The membrane was incubated with the primary Ab diluted in TBS-Tween/5% skim milk for 30 min at RT then washed three times for 10 min in TBS-Tween. The membrane was then incubated with secondary Ab; goat anti-mouse IgG-HRP (BioRad) diluted 1:3000 in TBS-Tween/5% skim milk, for 30 min at RT. After washing three times as before, the membrane was developed using ECL+plus system (Amersham Biosciences) according to the manufacture's instructions. The signal was detected by exposure to Fuji Super RX X-Ray film and quantified by phosho-imager analysis on a Storm 860 (Molecular Dynamics). The results were analyzed with the software program Image Quant for Macintosh version 1.2.

2.8 Indirect Immunofluorescence Assay

2.8.1 Cell Monolayer

Monolayers of HEL cells grown on 18 mm coverslips (Fisher Scientific) in a 12 well plate were fixed by washing twice with 1 ml PBS and incubating in 400 µl Fix solution (PBS with 5% formaldehyde, 2% sucrose) for 10 min at RT. The cells were then permeabilized by washing twice with 1 ml PBS and incubating in 400 µl Permeabilization solution (PBS with 0.6% NonidetP-40, 10% sucrose) for 10 min at RT. After washing twice more with 1 ml PBS/1% FBS, the cells were incubated with 100µl primary ICP4 Ab (1114, Goodwin Institute) diluted 1:1000 in PBS/1%FBS for 1hr at RT, and washed six times with PBS/1% FBS over 15 min. For the secondary Ab, the cells were incubated in 100µl Alexa Fluor® 488 goat anti-mouse IgG (Molecular Probes) diluted 1:1000 in PBS/1%FBS for 1 hr at RT and washed six times as before. The cell nuclei were stained by incubating in 100µl of 500 ng/ml Hoescht 33342 (Molecular Probes) in PBS solution for 10 min at RT, protected from the light. After washing three times in PBS/1% FBS, the coverslips were dipped in H_2O, and allowed to dry for 15 min at RT, protected from the light. The coverslips were mounted on the slides using 20µl Vectashield mounting media, and secured with clear nail polish.

2.8.2 Cell Suspension

Monolayers of HEL cells grown on 12 well plates were washed with 1 ml PBS and trypsinized with 500 µl trypsin until the cells were dissociated. The trypsin was inactivated by resuspending the cells in 500 µl DMEM/10%FBS. The media was removed by spinning at 2400 rpm in a Beckman G5-R swinging bucket centrifuge for 10 min at 4°C, and aspirating the supernatant. The cells were washed by resuspending in 400 µl PBS and centrifuging under the same conditions. To fix the cells, the pellet was resuspended in 400 µl fix solution (PBS with 5% formaldehyde, 2% sucrose) and incubated for 10 min at RT. Following one wash, the cells were permeabilized by resuspending in 400 µl permeabilization solution (PBS with 0.6% NP40, 10% sucrose) and incubating for 10 min at RT, and washed in 400 µl wash buffer (0.1% Tween in PBS). The cells were resuspended and blocked for 1 hr in 400 µl blocking buffer (2% goat serum, a 1:1000 dilution of Human Fc, and 0.1% Tween in PBS), then washed once in 400 µl wash buffer. Both the primary and secondary Abs were diluted in blocking buffer. Primary Abs used include: monoclonal Ab to U_S11 (Roller & Roizman, 1992), used at a 1:1000 dilution, and monoclonal Ab to gC (C3, donated by Dr. J. C. Glorioso), used at a 1:1000 dilution. A 1:1000 dilution of Alexa Fluor® 488 goat anti-mouse IgG (Molecular Probes) was used for the secondary Ab. Incubation with the primary and secondary Abs was done sequentially. For each of these steps, the cells were resuspended and incubated in 200 µl Ab solution for 1 hr at RT, then washed twice in wash buffer. Lastly, the cells were resuspended in 500 µl blocking buffer and transferred to a 5 ml Falcon tube for FACS analysis.

2.9 Northern Analysis

Monolayers of HEL cells grown in 60 mm plates were washed with 5 mls PBS, then incubated in 1 ml Trizol at RT for 5 min. A cell scraper was used to collect the cells for extraction in 0.2 ml chloroform per ml of Trizol. Following the addition of the chloroform, the mixture was shaken for 20 seconds by hand and centrifuged (Eppendorf centrifuge 5417C) at 11 000 rpm for 15 min at 4°C. The upper layer was transferred to a certified RNAse free eppendorf tube (Rose Scientific Ltd) and incubated with 0.5 ml isopropanol for 15 min at RT before centrifuging again under the same conditions. The pellet was washed with 0.5 ml 70% ethanol and centrifuged at 11 000 rpm for 5 min at 4°C. The supernatant was removed and the pellet was allowed to dry for about 5 min before resuspending in 30 µl DEPC water. The samples were stored at -80°C.

The RNA concentration of the samples was determined with an Ultrospec 3000 (Pharmacia) spectrometer at 260 nm. For each sample, 10 µg of RNA was mixed with 23 µl of loading buffer (3 µl of 10 X MOPS, 5 µl of formaldehyde, and 15 µl of formamide). 2 µg of RNA Millennium™ Size Markers (Ambion) was also mixed with 30 µl of loading buffer. The markers and samples were denatured for 15 min at 60°C and transferred to ice for 2 min before loading onto a 1% agarose, 2% formaldehyde, and 1X MOPS gel. The combs and gel tray used to make the gel were treated with RNaseZap® (Ambion). The gel was run for approximately 2 hrs at 100V in 1X MOPS running buffer. The gel was then stained with SYBR Gold Nucleic Acid Gel Stain (Molecular Probes) according the manufacturer's instructions and quantified by phosho-imager analysis on a Storm 860 (Molecular Dynamics). RNA was transferred to a GeneScreen Plus nylon membrane (NEN Life Sciences Products) in 10 X SSC (1.5 M sodium chloride, 150mM sodium citrate). The membrane was UV-cross linked using a Statalinker 2400 (Stratagene) before being hybridized to a ^{32}P-labeled probe. The ^{32}P signal was detected by exposure to Kodak BioMax MS film at -80°C and quantified by phosho-imager analysis on a Storm 860 (Molecular Dynamics). Results were analyzed with the software program Image Quant for Macintosh version 1.2.

2.10 Southern Analysis

Monolayers of HEL or U2OS cells grown on 12 well plates were washed once in 1 ml PBS, then incubated in 400 µl cell lysis buffer (10 mM Tris pH 7.5, 1 mM EDTA, 0.6 % SDS, 1 X protease K) for 3 hrs at 37°C. The DNA solution was extracted in equal volume pure phenol by rotating for 20 min at RT, and spinning (Eppendorf centrifuge 5417C) at 14 000 rpm for 15 min. The upper layer was extracted in chloroform using the same process then precipitated by mixing with 1/10 volumes 3M NaOAC and 2 volumes 95% ethanol and incubating for 1 hr at -80°C. After spinning at 14 000 rpm for 15 min at 4°C, the DNA was washed with 0.5 mls 70% ethanol and centrifuged at 14 000 rpm for 5 at RT. The liquid was aspirated and the DNA was allowed to dry for

1 hr before being resuspended in 65 µl Buffer P1 (Qiagen) and incubated at 37°C for 20 min. The samples were stored at -20°C.

10 µl of the DNA was restriction digested with *Bam* HI/*Nhe* I, *Sst* I/*Spe* I/*Pst* I, or *Pvu* II. Following restriction digest, the samples and 10 µl 1kbp DNA ladders (GeneRuler) were run on a 1 % agarose in Tris-acetate EDTA (TAE) gel for 2 hrs at 80 V in TAE buffer. The gel was then stained with SYBR Gold Nucleic Acid Gel Stain (Molecular Probes) according the manufacturer's instructions and quantified by phosho-imager analysis on a Storm 860 (Molecular Dynamics). The gel was washed sequentially in the following solutions for 15 min each: 0.25 M HCl, 0.5 M NaOH, 1 M Tris/1.5 M NaCl, and 10 X SSC. DNA was transferred to a GeneScreen Plus nylon membrane (NEN Life Sciences Products) in 10 X SSC. The membrane was UV-cross linked using Statalinker 2400 (Stratagene) before being hybridized to a ^{32}P-labelled probe. The ^{32}P signal was detected by exposure to Kodak BioMax MS film at -80°C and quantified by phospho-imager analysis on a Storm 860 (Molecular Dynamics). Results were analyzed with the software program Image Quant for Macintosh version 1.2.

2.11 Probes

2.11.1 Template DNA

Template DNA for ^{32}P-labeled probe was either amplified by PCR or isolated by restriction digest. The following sets of plasmids and primers or restriction enzymes were used to make the probes for this investigation:

DsRed2 probe: Plasmid: pDsRed2-C1 (CLONTECH)
Primer 1: GGG CCA CAA CAC CGT GAA GCT G
Primer 2: CGC GGT ACC GTC GAC TGC AG

603 bp probe fragment

EGFP probe: Plasmid: pEGFP-C1 (CLONTECH)

Primer 1: CAG ATC CGC TAG CGC TAC C

Primer 2: TTT CCA TGG ATC TAG ATC CGG TGG A

821 bp probe fragment

VP16 probe: Plasmid: pVP16-KOS (Weinheimer *et al.*, 1992)

Primer 1: CGC CGT CGG GCG TCC CAC AC

Primer 2: CGG GGG ATG CGG ATC CGG TCG CGC

1567 bp probe fragment

ICP0 probe: Plasmid: pSHZ (Nabel *et al.*, 1988)

Restriction enzymes: *Bam*H I/*Xho* I

431 bp probe fragment

TK probe: Plasmid: pTK173 (Varmuza & Smiley, 1985)

Restriction enzymes: *Sac* I/*Sma* I

662 bp probe fragment

ISG 56K probe: Plasmid: IMAGE clone 325364 for ISG-56K (Invitrogen)

Primer 1: CCC AGT CAC GAC GAC GTT GTA AAA CG

Primer 2: AGC GGA TAA CAA TTT CAC ACA GG

1325 bp probe fragment

2.11.2 ^{32}P labeling

2 µg of DNA random primers was mixed with 250 ng of template DNA in 25 µl H_2O. The DNA-primer mixture was denatured at 100°C for 5 min and

then placed on ice for 2 min. Random priming was initiated by the addition of 7 µl oligo labelling buffer, 2 µl BSA (10mg/mL), 5 µl α-^{32}P dCTP, and 1 µl 10 X Klenow buffer. After incubating at 37ºC for 30 min, the volume was adjusted to 100 µl by adding 50 µl TE (10mM Tris, pH 7.0, 1 mM EDTA), and the DNA was extracted with equal volume phenol chloroform. After spinning (Eppendorf centrifuge 5417C) at 14 000 rpm for 5 min at RT, the upper layer was removed and passed through a Nick column (Amersham Pharmacia Biotech) to remove unincorporated nucleotides. The probe was eluted in 500 µl TE, and the radioactive counts were determined using a Beckman LS 6500 scintillation detector.

2.11.3 Hybridization

Blots from Southern or Northern analysis were incubated with 6 mls prewarmed Express Hybridization Solution (BD Biosciences) at 68°C for 15 min while being rotated in a H1 16000 hybridization incubator (Tyler Research Instruments). The ^{32}P-labelled probe was denatured at 100°C for 5 min then cooled on ice for 2 min before it was added to the blot by mixing into the Express Hybridization Solution. The blot was incubated with the probe (1×10^7 counts per blot) for 1 hr at 68°C while being rotated in the hybridization oven. Following the hybridization, the blot was rinsed one time with 30 mls Express Hybridization Wash 1 solution (0.5% 20X SSC, 0.1% SDS), then washed two more times in the same solution for 20 min while being rotated at RT. The blot was washed three times in 30 mls Express Hybridization Wash 2 solution (10% 20X SSC, 0.05% SDS) for 15 min while being rotated at 50°C. Following the washes the blot was wrapped in saran wrap for analysis and stored at –20°C.

2.11.4 Oligonucleotide Probe

The GAPDH ^{32}P-labeled oligonucleotide probe was made and hybridized to the Northern blot described in Results Section 3.3 by R. Doepker using the West-Neat protocol as described in R. Doepker et al (Doepker *et al.*, 2004). GAPDH probe sequence: 5' TTG ACT CCG ACC TTC ACC TTC CCC AT 3'.

2.12 FACS Analysis

Monolayers of cells were prepared for FACS analysis by trypsinizing until the cells were dissociated from the growth surface, resuspending in DMEM and transferring to a 5 ml Falcon tube. Fluorescence was quantified by passing the cells through a Becton Dickson FACScan and analyzed using CellQuest Software.

2.13 Microscope Analysis

Light Microscope: Olympus CK40.

Fluorescent Microscope: Olympus BX40 (for slides), Leica DMIRB (for plates). A FITC filter was used for EGFP, and a Rhodamine filter was used for DsRed2. Pictures were taken with Spot Diagnostic Instruments Inc. camera using SPOT Advanced software.

Confocal Microscope; Zeiss LSM 510, 2 photon Laser Scanning Microscope system with two lasers giving excitation lines at 488 nm (for Alexa Fluor 488) and 780 nm (for Hoescht stain), and using a 40x oil immersion objective lens.

2.14 Plasmids

2.14.1 Production of pTK-Red and pTK-Green

The bacterial expression plasmid pTK173 (a gift from Dr. F. L. Graham, McMaster University) contains the HSV-1 thymidine kinase gene, ampicillin resistance genes, and PBR322 plasmid replication origin. To prepare pTK173 for an insertion into its thymidine kinase gene, the plasmid was linearized with *Sst* I and the ends were made blunt with T4 DNA polymerase.
The 1.6 kbp *Ase* I-*Mlu* I fragment of pEGFP-C1 (CLONTECH) and pDsRed2-C1 (CLONTECH) contains the human cytomegalovirus immediate early (CMV IE) promoter, the fluorescent gene *Discosoma sp.* Human codon-optimized Red Fluorescent protein (DsRed2) or enhanced green fluorescent protein (EGFP), a multiple cloning site (MCS), and a Simian virus 40 early mRNA polyadenylation (SV40 Poly A). These fragments were made blunt with the Klenow fragment of DNA polymerase I.

The pTK173 vector and insert fragment from pEGFP-C1 or pDsRed2-C1 were ligated together with DNA ligase. For a diagram of the resulting plasmids, pTK-Red and pTK-Green, see Figure 3.1.1. To ensure their accuracy, the plasmids pTK-Red and pTK-green were sequenced using the primers TK 5' (CTC ATA TCG GGG GGG AGG CTG GG) and TK 3' (AGG GCC GGG GGC GGG GCA TGT G) and a Beckman Coulter CEQ2000XL DNA Sequencing System at the Biochemistry DNA Services Laboratory, Faculty of Medicine, University of Alberta.

2.14.2 Amplification in *E. coli*

Plasmids were maintained and amplified in the *Escherichia coli (E. coli)* strain XL1-Blue (F$^-$: Tn10proA$^+$B$^+$lacI^{q}Δ(lacZ)MIS/recAIendAIgyrA96(NaI^r) thihsdRI7($r_K^-m_K^+$)glnV44relAIlac). Transformation of *E. coli* was achieved by combining 40 µl of XL1-Blue cells with 50 ng of plasmid DNA and pulsing at

1.8 of a BIORAD Gene Pulser II. Colonies transformed with the recombinant plasmids pTK-Red or pTK-Green were selected with ampicillin (100 µg/ml) on Luria-Bertani (LB: 1.0% bacto-tryptone, 0.5% yeast extract, 1.0% NaCl) agar plates (1.5% agar). Transformed colonies were cultured at 37°C in LB medium supplemented with 100 µg/ml of ampicillin while being shaken at approximately 250 rpm. Plasmids were isolated from bacterial culture using GenElute™ Plasmid Maxiprep kit according to the manufacturer's instructions. The concentration of the plasmids was determined with an Ultrospec 3000 (Pharmacia) spectrometer at 260 nm.

2.15 Production of Fluorescent Recombinant viruses

2.15.1 Preparation of infectious viral DNA

The virus was amplified by infecting a permissive cell line (grown in 150 cm^2 tissue culture flasks) with a MOI of 10 PFU/cell in 5 mls DMEM. The flasks were incubated at 37ºC for 1 hour, with rocking every 15 min. The innoculum was replaced with 25 mls DMEM, and the infected cells were incubated at 37ºC with 5% CO_2. After complete CPE was observed (24-48 h post-infection), the cells were centrifuged at 2700 rpm for 12 min at 4ºC in a swinging bucket centrifuge, and the supernatant was discarded. The cell pellet was washed in PBS then re-pelleted by centrifuging under the same conditions as before. After discarding the supernatant, the cells were lysed by the addition of 10% SDS. Protein was removed from the extracts first by overnight treatment with 100 µg/ml of proteinase K at 37ºC followed by four extractions with 4 ml of phenol/chloroform. The extract was dialyzed at 4ºC against 0.1 X SSC for 3 days, with the buffer changed twice daily. The infectious DNA was stored at 4ºC.

2.15.2 Co-transfection of U2OS cells

The pTK-Red or pTK-Green plasmids were restriction digested with the enzyme *Afl* III. *Afl* III has two restriction sites in the TK sequence of these plasmids: one located 400bp upstream and one 400bp downstream of the fluorescent insert fragment. 350 ng of the digested pTK-Red or pTK-Green plasmid was combined with 1300-2800 ng of infectious HSV DNA, 8 µl of Fugene (Roche), and 92 µl DMEM. The mixture was added drop-wise to monolayers of U2OS cells grown in 6 well plates (50-80% confluent), which were then incubated at 37ºC in a 5% CO_2 humidified atmosphere. After complete CPE was observed (5 to 7 days later), the cells were harvested, subjected to three freeze thaws and disrupted with a 550 Sonic Dismembrator. Cellular debris was removed by centrifugation at 2000 rpm for 10 min at 4ºC in a swinging bucket centrifuge and the supernatant was transferred to 5 ml falcon tubes and stored at –80ºC.

2.15.3 Plaque Purification

Virus stocks obtained from co-transfection were titrated using ten-fold dilutions ranging from 10^{-1} to 10^{-6} prepared in DMEM. 0.5 ml of each dilution was used to infect 6-well plates of confluent monolayers of U2OS cells. 1 hour post-infection the innoculum was replaced with 2 mls of DMEM supplemented with 10% FBS and 1% human immune serum, and 10 µg/mL acyclovir (Sigma). The plates were incubated at 37ºC and 5% CO_2 and plaques were allowed to develop. Isolated fluorescent plaques were identified using a fluorescent microscope with an FITC filter to illuminate EGFP, and a Rhodamine filter to illuminate DsRed2, and marked with a black Sharpie. The media was removed from wells with marked plaques and replaced with 2 mls of 1% agarose. The agarose was allowed to set for half an hour at RT, then plugs were picked from the marked area using a plastic micro-pipette tip, and stored in 0.28 ml DMEM.

The virus was released from cells in the plug by three rounds of freezing at -80ºC for 15 min and thawing in a 37ºC water bath. The samples were then amplified by infecting confluent monolayers of U2OS cells grown on 12-well plates. 1 hour post-infection the innoculum was replaced with 1 mL of DMEM/10% FBS and the plates were incubated at 37°C until CPE was observed. Plaque purification was repeated at least 3 times until no non-fluorescent virus was detected by titration of the stocks, and the recombinant viral DNA pattern (observed by Southern blotting) was pure.

CHAPTER 3 : RESULTS

3.1 Construction of fluorescent recombinant HSV-1 strains

Fluorescent recombinant strains of wild type HSV-1 strain KOS and the KOS mutants n212 (ICP0$^-$) (Cai & Schaffer, 1989) and KM110 (ICP0$^-$/VP16$^-$) (Mossman & Smiley, 1999) were constructed in order to develop an experimental system that would allow us to determine if ICP0 mutants are capable of productively infecting cells that harbour a quiescent viral genome. The plasmids pTK-Red and pTK-Green were first constructed. These plasmids contain genes encoding red fluorescence protein (DsRed2) and enhanced green fluorescence protein (EGFP), derived from the plasmids pDsRed2 and EGFP-C1 respectively, inserted into the vector plasmid pTK173 in the middle of the HSV-1 early gene thymidine kinase (TK). See Figure 3.1.1 for a diagram of these plasmids and Materials and Methods for details on their construction. U20S cells were co-transfected with the plasmids pTK-Red or pTK-Green and the infectious DNA of HSV-1 strains KOS, n212, or KM110. This allowed for the insertion of a fluorescence gene, under the control of the CMV E promoter, into the TK locus of the HSV genome by homologous recombination: disrupting the TK gene. The genome structures of the recombinant viruses KOS-Green (KOS-G), n212-Green (n212-G), and KM110-Red (KM110-R) are diagramed in Figure 3.1.2. The TK locus was chosen for the site of homologous recombination both because TK is nonessential in tissue culture (Roizman & Knipe, 2001) and because recombinant strains can be selected for in the presence of acyclovir. Acyclovir is a nucleoside analog that inhibits the replication of TK$^+$ HSV, but not of TK$^-$ virus (Elion *et al.*, 1977, Moolten, 1986). The specificity of acyclovir is a result of its dependency on phosphorylation by viral TK before it can be incorporated into newly synthesized DNA and result in chain termination (Elion *et al.*, 1977, Reardon & Spector, 1989).

3.1.1 Southern blot analysis

The recombinant viruses were subjected to at least three rounds of plaque purification (described in Materials and Methods) where recombinants were selected based on both acyclovir resistance and production of the fluorescent protein. Following plaque purification the recombinant viruses were analyzed by Southern blot and compared to parental strains in order to confirm their identity (Figure 3.1.4). KOS, n212, KM110, KOS-G, n212-G, and KM110-R were amplified on U2OS cells for 24 hrs before the infected cells were harvested and the DNA was isolated. DNA from each of the virus strains was allocated for five restriction digests: one for each of a *Bam*H I/*Nhe* I double digest and a *Sst* I/*Spe* I/*Pst* I triple digest, and three for *Pvu* II restriction digests. The samples were electrophoresed through agarose gels, then transferred to nylon membranes. The five resulting membranes (corresponding to each of the five restriction digests) were hybridized to ^{32}P-labelled probes for VP16, ICP0, TK, EGFP, or DsRed2 and exposed to X-Ray film.

Figure 3.1.3 illustrates the expected DNA fragment sizes arising from the VP16, ICP0 and TK loci for each of the virus strains. The wild type (wt) VP16 locus is borne by an 8.1 kbp *Bam*H I fragment which lacks internal *Nhe* I sites. In contrast, the V422 mutant (mt) form of the VP16 gene (Lam *et al.*, 1996) borne by KM110 and KM110-R bears a *Nhe* I restriction site, which is part of the chain terminating oligonucleotide used to construct the V422 mutation. Therefore, a *Bam*H I/*Nhe* I double digest is predicted to yield a single 8.1 kbp VP16 fragment from wt DNA (Figure 1.3A left), and two fragments of 4.9 and 3.2 kbp for mt DNA (Figure 1.3A right). The results of the Southern analysis show that, as expected, KOS, n212, KOS-G and n212-G all have an approximately 8.1 kbp fragment indicating a wt VP16 gene (Figure

1.4A lanes 2, 3, 5 and 6). In contrast, KM110 and KM110-R each have approximately 4.9 and a 3.2 kbp bands (Figure 1.4A lanes 4 and 7), indicating that they bear the *Nhe* I linker that marks the V422 VP16 mutation. The larger, 4.9 kbp band, is fainter than the 3.2 kbp band because the VP16 probe recognizes a much smaller portion of the 4.9 kbp fragment (Figure 1.3A left).

The wt ICP0 locus is borne by a 6.2 kbp *Sst* I/*Pst* I fragment which lacks internal *Spe* I sites. In contrast, the n212 mt form of the ICP0 gene (Cai & Schaffer, 1989) borne by n212, KM110, n212-G and KM110-R bears a *Spe* I restriction site, which is part of the chain terminating oligonucleotide used to construct the n212 mutation. Therefore, a *Sst* I/*Spe* I/*Pst* I triple digest is predicted to yield a single 6.2 kbp ICP0 fragment from wt DNA (Figure 1.3B left), and a single 2.4 kbp fragment from mt DNA (Figure 1.3B right). The results of the Southern analysis show that, as expected, KOS, and KOS-G have an approximately 6.2 kbp fragment indicating a wt ICP0 gene (Figure 1.4B lanes 2 and 5). In contrast, n212, KM110, n212-G and KM110-R all have an approximately 2.4 kbp band (Figure 1.4B lanes 3, 4, 6 and 7), indicating that they bear the *Spe* I linker that marks the n212 ICP0 mutation.

The wt TK locus is borne by a 2.0 kbp *Pvu* II fragment. In contrast, the EGFP mt form of the TK gene borne by n212-G and KOS-G bears a 1.7 kbp EGFP insert and the DsRed2 mt form of the TK gene borne by KM110-R bears a 1.6 kbp DsRed2 insert with an additional *Pvu* II site. Therefore, a *Pvu* II digest is predicted to yield a single 2.0 kbp TK fragment from wt DNA (Figure 1.3C top), a single 3.7 kbp fragment from EGFP mt DNA (Figure 1.3C bottom left), and 1.9 and 1.7 kbp fragments from DsRed2 mt DNA (Figure 1.3C bottom right). The results of the Southern analysis show that, as expected, KOS, n212, and KM110-R all have an approximately 2.0 kbp fragment indicating a wt TK gene (Figure 1.4C lanes 2, 3, and 4). In contrast, n212-G and KOS-G have an approximately 3.7 kbp band recognized by both the TK probe (Figure 1.4C lanes 5 and 6) and the EGFP probe (Figure 1.4D lanes 5 and 6), indicating that they bear the EGFP mutation. KM110-R has approximately 1.9 and 1.7 kbp bands recognized by both the TK probe (Figure 1.4C lane 7) and DsRed2 probe (Figure 1.4D lane 7), indicating that it bears the DsRed2 mutation. The smaller, 1.7 kbp band, is fainter than the 1.9 kbp band because the DsRed2 probe recognizes a much smaller portion of the 1.7 kbp fragment (Figure 1.3C bottom right). The DsRed2 probe also hybridized to the 3.7 kbp fragment of KOS-G and n212-G (Figure 1.4E lanes 5 and 6) indicating there is some sequence similarity between the EGFP and DsRed2 genes.

The results from this Southern analysis establish that the recombinant viruses made for this investigation have the expected mutations and that the stocks are free from contaminating virus.

3.1.2 Transgene expression

In order to determine if the EGFP and DsRed2 transgenes borne by the recombinant strains were expressed, we tested for the production of fluorescent protein during infection. Plaques resulting from infection of U2OS cells (a permissive cell line for ICP0 and ICP0/VP16 double mutants (Mossman & Smiley, 1999, Yao & Schaffer, 1995)) with the recombinant viruses were observed under a fluorescent microscope. The CMV IE promoter driving the fluorescent genes has previously been shown to be strongly expressed in many mammalian cell lines (Foecking & Hofstetter, 1986). We found that by one day post-infection the fluorescent proteins could be detected and were strongly expressed from the red or green recombinant KOS, n212 and KM110 strains. See figure 3.1.5 for photographs of fluorescent plaques resulting from n212-G or KM110-R infection of U2OS cells.

3.2 ICP0 is a specific requirement for the reactivation of DNA replication and gene expression of quiescent HSV-1

The HSV IE gene product ICP0 has been shown to be necessary and sufficient for the reactivation of a quiescent genome (Everett *et al.*, 1998b, Harris & Preston, 1991, Stow & Stow, 1989). In order to confirm and extend these findings in our experimental system, we asked if the lack of any HSV-1 products other than ICP0 prevents reactivation of a quiescent genome. The ability of HSV-1 strains mutant in the IE genes ICP0 (n212 (Cai & Schaffer, 1989)), ICP4 (d120 (DeLuca *et al.*, 1985)), ICP22 (d22lacZ (Long *et al.*, 1999)), and ICP27 (d27-1 (Rice & Knipe, 1990)) to reactivate quiescent KM110-R was tested and compared to wild type (KOS).

Confluent monolayers of HEL cells were either mock treated or infected with KM110-R at a multiplicity of infection (MOI) of 2 (Figure 3.2A) or 6 (Figure 3.2B and C) in order to establish a quiescent infection. Even with a high MOI of KM110, such as 10, the virus persists in a quiescent but inducible state in HEL cells for at least 10 days, during which time no viral proteins are produced and the cells remain healthy (Mossman & Smiley, 1999). Four days later the cells were either mock treated, or superinfected (SI) with an MOI of 10 of wild type or an IE mutant. The samples were harvested the next day and analyzed by FACS, Southern, and Western for evidence of reactivation of the quiescent genome.

3.2.1 Reactivation of the DsRed2 transgene

FACs analysis was used to detect the expression of the IE gene product DsRed2 from KM110-R (Figure 3.2A). For these samples, the cells were harvested by trypsinizing and suspending in cell culture medium, then analyzed by FACS to detect production of fluorescence. The results are presented as dot plots with increasing red fluorescence on the y-axis and increasing green fluorescence on the x-axis. Preliminary experiments were done in order to establish boundaries for cells expressing DsRed2, EGFP, both DsRed2 and EGFP, or no fluorescence. These boundaries were then used to determine the proportion of cells in each grouping.

We found that only a small percentage (1%) of KM110-R infected cells expressed DsRed2 from the KM110-R genome (Figure 3.2A box 1). In contrast, SI with KOS resulted in 28% of cells expressing DsRed2 (Figure 3.2A box 2), indicating these cells harbour KM110-R that can be reactivated by wt virus. The proportion of cells expressing DsRed2 following SI with KOS can be used as a minimal estimate of the proportion of cells harbouring the KM110-R (discussed in more detail in Section 3.4). Therefore, the proportion of cells known to harbour KM110-R (28%) compared to the small proportion of cells expressing DsRed2 in the absence of wt SI (1%) indicates that KM110-R is quiescent in HEL cells; a result that is consistent with previous experiments involving KM110 (Mossman & Smiley, 1999). SI with d120, d22lacz and d27-1 also led to 28%-44% of cells expressing DsRed2 (Figure 3.2A boxes 4, 5 and 6), indicating that these IE mutants are also able to reactivate a quiescent genome. ICP4 mutants do not express early (E) or late (L) gene products (DeLuca *et al.*, 1985); therefore, the ability of this mutant to reactivate KM110-R precludes the possibility that E or L gene products are necessary for reactivation.

An accurate determination of the proportion of cells harbouring the KM110-R genome is essential for drawing meaningful conclusions from our experiments. We were concerned that production of progeny KM110-R virus and their spread to neighbouring cells following reactivation by the SI virus would lead to an overestimate of the proportion of cells initially infected by KM110-R. We therefore tested the ability of each of the SI viruses to prevent subsequent infection of cells with progeny virus. We found that previous infection with wild type virus (Figure 4.2) or an ICP0, ICP22, or ICP27 mutant (data not shown) prevents the SI of cells (discussed in more detail in Section 3.4). In contrast, ICP4 mutants are less able to prevent SI (data not shown), and may allow the spread of progeny KM110-R into nearby cells following reactivation of the quiescent genome. Therefore, the higher percent of cells expressing DsRed2 following d120 SI may be due to the inability of d120 to fully prevent SI.

Only 1% of cells SI with n212 expressed DsRed2 from the KM110-R genome (Figure 3.2A box 3), a result virtually identical to mock treatment. This result is consistent with previous studies that have shown that ICP0 mutants are incapable of reactivating a quiescent genome (Harris *et al.*, 1989).

The results of this FACs analysis show that the IE gene products ICP22, ICP27, and ICP4 (and therefore any E or L gene products) are not necessary for the reactivation of quiescent KM110-R and imply that ICP0 alone is necessary for reactivation of a quiescent genome.

3.2.2 Reactivation of viral DNA Replication

Previous reports implied that ICP0 is required for the quiescent viral genome to replicate in superinfected cells (Harris *et al.*, 1989). Inasmuch as ICP0 mutants themselves are capable of replication following high MOI infection, the implication is that quiescent genomes are inaccessible to the viral DNA replication machinery. We therefore used Southern blot hybridization to monitor replication of the resident quiescent genome following SI with wt HSV-KOS and the immediate early mutants described above (Figure 3.2B). For these samples, the DNA was *Bam*H I/*Nhe* I restriction digested, electrophoresed through an agarose gel, and transferred to a nylon membrane. The membranes were hybridized to a ^{32}P-labeled probe for VP16 and exposed to X-Ray film. Replication of the KM110-R genome can be distinguished from replication of the SI genome by the presence of the *Nhe* I linker in the VP16 locus of strains with a KM110 background.

Cells infected with KM110-R did not have detectable 4.9 and 3.2 kbp bands (Figure 3.2B lane 2), indicating that the copy number of viral DNA was below the detection limit of this assay under the conditions used. However, bands corresponding to the KM110-R VP16 DNA were detected following SI with KOS (Figure 3.2B lane 4), demonstrating that the quiescent genome was amplified in response to infection. Similarly, SI with d120, d22lacZ or d27-1 also lead to the amplification of the KM110-R genome (Figure 3.2B lanes 8, 10, and 12). In contrast, SI with n212 did not lead to detectable amplification of the quiescent genome (Figure 3.2B lane 6). The results of this Southern analysis are consistent with the previously described FACs analysis and indicate that ICP0 mutants alone are unable to reactivate the quiescent KM110-R genome. In fact, the IE mutants that cannot replicate on HEL cells alone, such as ICP4 (Figure 3.2B lane 7) (Preston, 1979) and ICP27 (Figure 3.2B lane 11) (Sacks *et al.*, 1985) mutants, show increased replication on monolayers that harbour KM110-R (Figure 3.2B lanes 8 and 12: 8.1 kbp band). This indicates that reactivation of the quiescent KM110-R genome provides the missing, necessary, proteins for the SI virus to proceed with DNA replication. Replication of the superinfecting ICP0 mutant genome, on the other hand, appears to be reduced on monolayers where many of the cells harbour KM110-R (Figure 3.2B lane 6) compared to cells with no KM110-R (Figure 3.2B lane 5), indicating that quiescent infection by KM110-R hinders the replication of ICP0 mutants. These results are consistent with those found by Dr. Mossman in our lab while conducting similar experiments using KM110 (Mossman, unpublished data). Therefore, further experiments are needed to determine if ICP0 mutants are able to replicate in cells that harbour the KM110-R genome.

3.2.3 Reactivation of the expression of the VP16 gene

For further confirmation of the results obtained by Southern analysis we also used Western analysis to monitor VP16 production from the resident quiescent genome following SI with wt HSV-KOS and the immediate early mutants described above (Figure 3.2C). The V422 VP16 mutation (Lam *et al.*, 1996) is marked by a stop codon that leads to a truncation of VP16 after amino acid residue 422. Therefore, the mutant (mt) VP16 expressed from the reactivated KM110-R genome is smaller (Mossman & Smiley, 1999) and can be distinguished from wild type (wt) VP16 expressed from the SI virus. VP16 is a leaky late protein that can be produced in the absence of DNA replication (Roizman & Knipe, 2001). Therefore, we also tested for the production of the true late protein U_S11, which is produced only if DNA replication has occurred (Roizman & Knipe, 2001); however, the production of U_S11 from KM110-R and the SI virus cannot be distinguished. Additionally, an Ab to the cellular protein actin was used as a loading control. For these samples, the isolated proteins were electrophoresed through a 12% acrylimide gel, and transferred to a nitrocellulose membrane. The membranes were incubated with primary antibodies to VP16, U_S11, and actin, then with an HRP-labeled secondary antibody. Following incubation with the secondary antibody, the membrane was treated with ECL plus, and exposed to X-Ray film.

Cells infected with KM110-R did not show production of mt VP16 or U_S11 (Figure 3.2C lane 2), indicating that the expression of these proteins was below the detection limit of this assay under the conditions used. However, production of mt VP16 was detected following SI with KOS (Figure 3.2C lane 4), demonstrating that the quiescent genome was reactivated in response to SI. Similarly, SI with d120, d22lacZ or d27-1 also lead to the production of mt VP16, indicating reactivation of the KM110-R genome (Figure 3.2C lanes 8, 10, and 12). In contrast, SI with n212 did not lead to production of mt VP16 by KM110-R (Figure 3.2C lane 6). Similarly to the results seen from Southern analysis, the production of U_S11 and VP16 by the ICP4 and ICP27 mutants were increased on monolayers that harboured KM110-R (Figure 3.2C lanes 8 and 12). The results of this Western analysis are consistent with the previously described FACS and Southern analysis and indicate that ICP0 alone is necessary for the reactivation of a quiescent genome.

3.3 The antiviral response induced by KM110-Red dissipates and has a diminished effect on wild-type virus by four days post-induction

Infection of HEL cells with KM110 leads to the induction of many of the same genes as treatment with IFN (Mossman *et al.*, 2001). Additionally, ICP0 mutants have been shown to be hypersensitive to the effects of IFN (Mossman *et al.*, 2000). As mentioned in the previous section, replication of the ICP0 mutant genome appears to be reduced on monolayers that had previously been infected with KM110-R (Figure 3.2B lane 6) compared to cells that had been mock treated (Figure 3.2B lane 5). Therefore, it seemed likely that replication of the ICP0 mutant was hindered by an antiviral response induced by KM110-R. In order to determine if the antiviral response was likely to affect the SI virus four days post-induction, we used Northern analysis to look for evidence of a persisting antiviral response, and a plaque reduction assay to test for any effect on wild type virus replication.

Northern analysis was used to test for the production of ISG 56K mRNA over the course of four days post-KM110-R infection of HEL cells (Figure 3.3.1). As described in the introduction, ISG 56 K is an interferon stimulated gene that is induced following KM110 infection of HEL cells (Mossman *et al.*, 2001). In two independent experiments, HEL cells were treated with 1000

U/mL IFN-α, mock treated or infected with a MOI of 6 of KM110-R. IFN-α treatment was used as a positive control for ISG 56K induction. The samples were harvested for RNA one to four days post-treatment, electrophoresed through an agarose gel and transferred to a nylon membrane. The membrane was hybridized using an ISG 56K probe and developed onto X-Ray film (Figure 3.3.1A top). The results were quantified using phosphor-imager analysis. After stripping the blots, and waiting a number of weeks for the ISG 56K signal to completely disappear, the blots were re-probed for cellular GAPDH (Figure 3.3.1A bottom) by R. Doepker in our lab and quantified using phosphor-imager analysis. The ISG 56K signal was normalized to the GAPDH signal and the average signal of the two independent experiments was plotted against each time point (Figure 3.3.1B). Consistent with results found by Mossman and colleagues using KM110 (Mossman *et al.*, 2001), we found that ISG 56K was induced one day post-infection by KM110-R (Figure 3.3.1A lane 3). Our results indicate that the level of ISG 56K induced by KM110-R drops sharply. Two days post-infection the level of ISG 56K is, on average, only 28% of what was seen one day post-infection (Figure 3.3.1B KM110-R). The levels continue to decline to 22% on day three, then to 13.5% on day four post-infection (Figure 3.3.1B KM110-R). This experiment also shows there is very little induction of ISG 56K by mock treatment. The level of ISG 56K induction in the mock infected HEL cells was highest on day three, but was only 5% (on average) of what was induced by KM110-R on day 1 (Figure 3.3.1B Mock). The results of this Northern indicate that the antiviral response induced by KM110-R upon infection dissipates by four days post-infection. However, the residual antiviral response may still have an affect on viral replication. Additionally, expression of other ISGs induced by KM110-R may not have dissipated by four days post-infection. Therefore, we used a plaque reduction assay for a more direct test of whether viral replication was affected four days post-induction of the antiviral response.

A plaque reduction assay was used to test the ability of a low MOI of wild type HSV to replicate on HEL cells one and four days post-induction of an antiviral state by infection with KM110-R. In two independent experiments, HEL cells were either mock treated or infected with a MOI of 6 of KM110-R. One day and four days later, the samples were either mock treated or SI with 2.7 x 10^3 PFU KOS (an MOI of approximately 0.005-0.01) and incubated in cell culture medium containing 0.5% methylcellulose for five days before being Giemsa stained. We found that, on one day post-treatment, infection of mock-treated HEL cells with KOS results in almost total destruction of the monolayer (Figure 3.3.2A top right). Consistent with results found by Mossman and colleagues using KM110 (Mossman *et al.*, 2001), we found that, on one day post-treatment, SI of KM110-R infected monolayers with KOS results in little destruction of the monolayer (Figure 3.3.2A bottom right). This marked decrease in plaque size and number indicates that, at a low MOI, KOS is severely inhibited by the antiviral response induced by KM110-R one day post-infection. The plaques formed by infection of HEL cells with KOS four days post-mock treatment (Figure 3.3.2B top right) are smaller than those formed by infection of HEL cells with KOS one day post-mock treatment (Figure 3.3.2A top right). This is most likely due to inhibition of viral replication on an older, over-confluent monolayer. The plaques formed on HEL cells SI with KOS four days post-KM110-R infection (Figure 3.3.2B bottom right) are smaller than plaques formed on HEL cells four days post-mock-treatment (Figure 3.3.2B top right), indicating that the antiviral response inhibits KOS replication even four days post-induction. However, more and slighty larger plaques are seen for monolayers that were SI with KOS four days post-KM110-R infection (Figure 3.3.2B bottom right) compared to one-day post-KM110-R infection (Figure 3.3.2A bottom right). This result is consistent with the decrease of ISG 56K mRNA seen by the previously described

Northern, and indicates that the antiviral response is not as effective four days post-induction.

Increasing the MOI of the SI virus was shown to help KOS overcome the effects of the antiviral response in repeat experiments of the plaque reduction assay (data not shown). Additionally, the Southern analysis described in section 3.2 indicates that, at a MOI of 10, replication of the wild type virus genome is not severely decreased on monolayers previously infected with KM110-R compared to mock treated monolayers (Figure 3.2B lanes 3 and 4). However, the antiviral response induced by KM110-R is expected to have a much greater effect on ICP0 mutants because, as mentioned earlier, they are much more sensitive to IFN than wild type virus. Therefore, in order to compensate for the hypersensitivity of ICP0 mutants to the antiviral response induced by KM110-R, a MOI of 30 of n212 was used instead of a MOI of 10 for subsequent experiments.

3.4 ICP0 mutants are able to superinfect HEL cells harbouring the quiescent KM110-Red genome

We next asked if ICP0 mutants are able to overcome the antiviral response induced by KM110-R and infect cells that harbour the KM110-R genome. FACS analysis of HEL cells infected with KM110-R and SI with wild type, an ICP0 mutant, or the fluorescent recombinants of each, was used to determine the proportion of cells infected with each virus (Figure 3.4.1). In four independent experiments, HEL cells were infected with a MOI of 6 of KM110-R. This MOI of KM110-R was chosen because preliminary experiments showed that at a MOI of 6 the KM110-R genome was largely silent, but close to half of the cells harboured the quiescent genome. Higher MOIs of KM110-R led to increased expression from the KM110-R genome, while lower MOIs lead to a smaller proportion of cells harbouring the quiescent genome. Four days later the cells were mock treated or SI with a MOI of 30 of n212 or n212-G or a MOI of 10 of KOS or KOS-G. The cells were harvested for FACS analysis eighteen hours post-SI. The results are presented as dot plots with increasing red fluorescence on the y-axis and increasing green fluorescence on the x-axis. The value reported for each sample includes the average of four independent experiments and the standard deviation.

In these experiments we found that, consistent with the previously described FACS analysis (Section 3.2), KM110-R is largely quiescent in HEL cells as evidenced by the small percent (6.5% ± 4%) of cells expressing red fluorescence following mock treatment of cells harbouring KM110-R (Figure 3.4.1A). The proportion of cells harbouring quiescent KM110-R was therefore determined by superinfecting with wild type virus, which leads to reactivation of KM110-R. We found that 45.3% ± 4% of cells expressed red fluorescence following KOS SI four days after a MOI of 6 infection of KM110-R, indicating that these cells harbour KM110-R (Figure 3.4.1C). However the proportion of cells ascertained to harbour the quiescent genome may be inflated due to replication of KM110-R and subsequent infection of adjacent cells following SI with wild-type virus. In order to evaluate this possibility we assessed the ability of HSV-1 infected cells to exclude superinfecting virus. In two independent experiments, monolayers of HEL cells were mock treated or infected with a MOI of 10 of KOS. After 12 hrs, the cells were SI with a MOI of 10 of KOS-G. Fifteen hrs post-SI (27 hrs post-infection) the samples were harvested and analyzed by FACS. We found that productively infected cells are immune to superinfection, as shown by the inability of the KOS-G to infect and express green fluorescence in the cells that had previously been infected with KOS (Figure 3.4.2 right). This result is consistent with previous experiments that have shown that expression of gD on cellular surface membranes, which normally occurs during HSV infection, prevents HSV from superinfecting cells that are already productively infected (Campadelli-Fiume *et* *al.*, 1988, Campadelli-Fiume *et al.*, 1990, Dasika & Letchworth, 2000). Infecting HEL cells with KM110-R and SI with KOS-G four days later lead to 97.4% ± 2% of cells expressing EGFP from the KOS-G genome (Figure 3.4.1E), indicating that wild type virus is able to infect almost every cell. Therefore, the spread of progeny KM110-R into nearby cells following SI with wild type virus is prevented. From this, we can conclude that the proportion of cells expressing red fluorescence from the KM110-R genome following SI with KOS represents the cells initially infected with KM110-R.

Only 4.4% ± 3% of cells infected with KM110-R and SI with n212 expressed DsRed2 from the KM110-R genome (Figure 3.4.1B). This result is very similar to mock treatment and confirms that, even with an increased MOI, SI with an ICP0 mutant does not lead to reactivation of the quiescent genome. 97.4% ± 2% of KM110-R infected cells SI with n212-G expressed EGFP from the n212-G genome (Figure 3.4.1D). As described previously, following a MOI of 6 infection with KM110-R, 45.3% ± 2% of these cells harbour KM110-R (Figure 3.4.1C). Therefore, as a minimal estimate, 42% ± 6% of cells infected with n212-G in this experiment harbour the quiescent KM110-R genome; demonstrating that ICP0 mutants are capable of infecting cells that harbour KM110-R. However, the expression of the IE gene EGFP does not indicate that ICP0 mutants are able to overcome the antiviral response and complete the lytic cycle. Therefore, further experiments were done in order to determine if an ICP0 mutant is capable of replicating in cells that harbour KM110-R.

3.5 Replication of ICP0 mutants in HEL cells does not lead to reactivation of the quiescent KM110-Red genome

Previous studies have shown that stalled infection following the expression of some IE gene products is a phenotype of ICP0 mutants (Cai & Schaffer, 1992) (Everett *et al.*, 2004a). Therefore, the expression of the E gene product EGFP (as was seen by FACS analysis in the preceding section) does not necessarily indicate a productive infection. In order to determine if ICP0 mutants are capable of proceeding past the IE stage following infection of cells harbouring quiescent KM110-R, we looked for evidence of the formation of viral DNA replication compartments using indirect immunofluorescence assays (IFA), and gene expression using Southern, Western and progeny assays.

3.5.1 Viral DNA replication compartments

In three independent experiments, HEL cells grown on coverslips were mock treated or infected with a MOI of 6 of KM110-R. Four days post-infection the cells were mock treated or SI with a MOI of 30 of n212 or a MOI of 10 of KOS in the presence or absence of 400 µg/mL phosphonoacetic acid (PAA). Preliminary experiments indicated that 9 1/2 hrs post-SI the replication compartments of ICP0 mutants were most distinct. At earlier times points the replication compartments are too small to be readily distinguished from non-productive infection, and at later time points the replication compartments are less distinctive as they fill the entire nucleus. Therefore, 9 1/2 hrs post-SI the samples were fixed and treated for indirect IFA of cell monolayers (Materials and Methods 2.8.1) using a primary Ab to ICP4, an Alexa Fluor 488 (green fluorescent) secondary Ab, and Hoescht for nuclei stain. For quantification, fluorescent microscope pictures were taken of each sample. From these, 100-650 cells were scored as either positive or negative for replication compartments. Samples where DNA replication was prevented by treatment with PAA were used as a guide for a non-productive infection and cells that did not have distinct replication compartments were considered negative. The value reported for each sample includes the average of three independent experiments and the standard deviation.

We first tested if using a primary Ab for ICP4 would allow us to distinguish between productively and nonproductively infected cells. ICP4 is an IE gene product and is expressed whether viral DNA replication occurs or not. As described previously in the introduction, at early times post-infection ICP4 is found diffuse throughout the nucleus (Knipe *et al.*, 1987) while later on in infection it is redistributed to globular replication compartments, within which it has a somewhat diffuse distribution (de Bruyn Kops *et al.*, 1998). The redistribution of ICP4 to replication compartments is dependent upon viral DNA replication (Knipe *et al.*, 1987). Therefore, we compared KOS or n212 infected samples where DNA replication was prevented using PAA with samples where infection was allowed to proceed normally. We found that samples treated with PAA had diffuse expression of ICP4 throughout the nucleus (Figure 3.5.1A left top and bottom) and were easily distinguishable from samples where DNA replication was allowed to proceed and had distinct, globular, replication compartments (Figure 3.5.1A right top and bottom). The brightly staining foci seen in samples treated with PAA (Figure 3.5.1A left top and bottom) may be areas adjacent to ND10 where ICP4 has been recruited to the viral genome (Everett *et al.*, 2004b).

KM110-R infected cells had only 2% ± 2% (991 cells scored) of cells score positive for viral DNA replication compartments (Figure 3.5.1B middle and right). This result is consistent with the previously described FACS, Southern and Western data (Sections 3.2 and 3.4) and indicates that KM110-R is largely quiescent in HEL cells. In contrast, following KOS SI, viral DNA replication compartments were formed in 97% ± 2% (1069 cells scored) of mock treated cells and 97% ± 2% (866 cells scored) of KM110-R infected cells (Figure 3.5.1D). Originally, we SI mock treated and KM110-R infected cells with a MOI of 10 of n212. However, with this MOI we found that viral DNA replication compartments formed in only 45% of cells (data not shown). Therefore, we used a higher MOI of n212 in order to increase the likelihood of the ICP0 mutant proceeding through the lytic cycle. At a MOI of 30, n212 was able to form replication compartments in 92% ± 2% (1578 cells scored) of mock treated cells (Figure 3.5.1C left), and 88% ± 2% (1661 cells scored) of KM110-R infected cells (Figure 3.5.1C right). The FACS analysis described in section 3.4 demonstrated that, with a MOI of 6 of KM110-R, 45% ± 4% of cells harbour the KM110-R genome. This, taken together with the finding that viral DNA replication compartments formed in 88% ± 2% of KM110-R treated cells following n212 SI, allows us to determine, as a minimal estimate, that 33% ± 6% of the cells SI by the ICP0 mutant harboured the quiescent KM110-R genome. This indicates that, at a high enough MOI, ICP0 mutants are capable of overcoming the antiviral response induced by KM110-R infection and forming viral DNA replication compartments.

3.5.2 DNA replication, gene expression, and production of progeny virus

Although indirect IFA showed that, at a high MOI, ICP0 mutants are able to productively infect cells that harbour the quiescent KM110-R genome, we could not determine from these experiments if the KM110-R genome remained silent under these conditions. We therefore compared the amounts of DNA replication, expression of late gene products and production of progeny resulting from the KM110-R genome following SI with wild type virus or an ICP0 mutant. We also compared the expression of these viral products from the SI genome in cells that had been infected with KM110-R to cells that had been mock treated. This allowed us to determine if the antiviral response induced by KM110-R inhibited productive infection of the SI virus. In four independent experiments, HEL cells were mock treated or infected with a MOI of 6 of KM110-R. Four days later the cells were mock treated, or SI with a MOI of 30 of n212 or a MOI of 10 of KOS. Eighteen hrs post-SI the cells were harvested for analysis by Southern, Western or progeny assay. Samples of cells SI with a MOI of 10 of KOS or a MOI of 30 of n212 were also harvested 1 hr post-SI in order to confirm that viral products brought in with the virion were negligible compared to the amount of newly synthesized viral products in these experiments. Additionally, samples used for the progeny assay were Acid/Glycine washed (described in Materials and Methods) following SI in order to remove unabsorbed virions from the surface of the cells.

3.5.2.1 DNA replication

Southern analysis of the VP16 locus was used to test for replication of the KM110-R and SI virus genomes (Figure 3.5.2). For these samples, the DNA was *Bam*H I/*Nhe* I restriction digested, electrophoresed through an agarose gel, and transferred to a nylon membrane. The membranes were then hybridized using a ^{32}P-labeled VP16 probe and exposed to X-Ray film. We tested samples harvested 1 hr post-infection with a MOI of 10 of KOS or MOI of 30 of n212 in order to determine if we could detect viral DNA brought in with the virion. We found that DNA from the virion was not detectable for KOS (Figure 3.5.2A lane 8) and barely detectable for n212 (Figure 3.5.2A lane 9) and therefore would not affect our results. As previously described, replication of the reactivated quiescent KM110-R genome can be distinguished from replication of the SI genome by the presence of the *Nhe* I linker in the VP16 locus of strains with a KM110 background. The signal intensities of the 8.1 kbp band resulting from replication of the SI genome and the 4.9 kbp and 3.2 kbp bands resulting from replication of the KM110-R genome were separately quantified using phospho-imager analysis. These values were then normalized to the amount of total DNA for each sample and reported as a percent relative to KOS SI of mock treated cells (for wild type VP16, Figure 3.5.2B) or as a percent relative to KOS SI of KM110-R infected cells (for mutant VP16, Figure 3.5.2C). Additionally, the values obtained following quantification of the 4.9 kbp and 3.2 kbp band area from samples that had been mock treated before SI were subtracted from the value obtained from samples that had been KM110-R infected before SI. This was done in order to remove background signal and thereby give a more accurate portrayal of the signal resulting from replication of the KM110-R genome. The value reported for each sample includes the average of four independent experiments and the standard deviation.

Following n212 SI, we found that the amount of DNA replication from the n212 genome was 84% ± 14% on mock treated cells, and 80% ± 21% on KM110-R treated cells: relative to wild type virus replication (Figure 3.5.2B n212). The lack of a significant decrease in the amount of n212 DNA produced from cells where 45.3% ± 4% of cells harbour KM110-R (FACS analysis, Section 3.4) indicates that the ICP0 mutant is not excluded from replicating its genome in these cells. This result confirms the previously described indirect IFA data and indicates that, at a high MOI, the ICP0 mutant is able to productively infect cells that harbour KM110-R. We found that replication of the KOS genome was inhibited to some extent on cells that had previously been infected with KM110-R compared to cells that had been mock treated. The amount of replication of the KOS genome on KM110-R infected cells was 75% ± 16% compared to 100% on mock treated cells (Figure 3.5.2B KOS). SI with a higher MOI of n212 did not lead to reactivation of and DNA replication from the quiescent KM110-R genome, as indicated by the absence of 4.9 kbp and 3.2 kbp bands (Figure 3.5.2A lane 4). This confirms that, even under conditions where productive infection of the SI virus occurs, the quiescent genome is not reactivated in the absence of ICP0. In contrast, following SI with KOS, the quiescent KM110-R genome was reactivated, as indicated by the production of 4.9 kbp and 3.2 kbp bands (Figure 3.5.2A lane 6).

3.5.2.2 Late gene expression

Western analysis of late gene products was used to test for expression of VP16 and U_S11 from the KM110-R and SI virus genomes (Figure 3.5.2). For these samples, the isolated proteins were electrophoresed through a 12% acrylimide gel, and transferred to a nitrocellulose membrane. The membranes were incubated with primary antibodies to VP16, U_S11, and actin, then with a HRP labeled secondary antibody. Following incubation with the secondary antibody, the membrane was treated with ECL plus, and developed onto X-Ray film. We tested samples harvested 1 hr post-infection with a MOI of 10 of KOS or MOI of 30 of n212 in order to determine if we could detect proteins brought in with the virion. We found that VP16 and U_S11 from the virion were not detectable for KOS (Figure 3.5.2A lane 8) and were barely detectable for n212 (Figure 3.5.2A lane 9) and therefore would not affect our results. As previously described, VP16 expressed from the reactivated quiescent KM110-R genome can be distinguished from VP16 expressed from the SI genome by the presence of the *Nhe* I linker in the VP16 locus of strains with a KM110 background, which leads to the truncation of VP16. The signal intensities of U_S11, the wild type (wt) VP16 resulting from expression of the SI genome and the mutant (mt) VP16 resulting from expression of the KM110-R genome were separately quantified using phospho-imager analysis. These values were then normalized to the amount of actin and reported as a percent relative to KOS SI of mock treated cells (for wt VP16 and U_S11, Figure 3.5.3B and D) or as a percent relative to KOS SI of KM110-R infected cells (for mt VP16, Figure 3.5.3C). Additionally, the values obtained following quantification of the mt VP16 band area from samples that had been mock treated before SI were subtracted from the value obtained from samples that had been KM110-R infected before SI. This was done in order to remove background signal and thereby give a more accurate portrayal of the signal resulting from expression of mt VP16 from the KM110-R genome. The value reported for each sample includes the average of four independent experiments and the standard deviation.

Following n212 SI, we found that the amount of wild type VP16 expressed from the n212 genome was 98% ± 21% on mock treated cells, and 104% ± 16% on KM110-R treated cells: relative to wild type virus expression of VP16 (Figure 3.5.3B n212). Similarly, the amount of U_S11 produced following n212 SI was 99% ± 14% on mock treated cells and 90% ± 15% on KM110-R treated cells (Figure 3.5.3D n212). The lack of a significant decrease in the amount of late gene products expressed from n212 on cells where 45.3% ± 4% of cells harbour KM110-R (FACS analysis, Section 3.4) indicates that the ICP0 mutant is not excluded from expressing late gene products in these cells. This result confirms the previously described indirect IFA and Southern data and indicates that, at a high MOI, the ICP0 mutant is able to productively infect cells that harbour KM110-R. We found that the expression of late gene products from the KOS genome was inhibited to some extent on cells that had previously been infected with KM110-R compared to cells that had been mock treated. The amount of VP16 expressed from the KOS genome on KM110-R infected cells was 83% ± 9% compared to 100% on mock treated cells (Figure 3.5.3B KOS). Similarly, the amount of U_S11 produced following KOS SI was 73% ± 14% on KM110-R treated cells compared to 100% on mock treated cells (Figure 3.5.3D n212). SI with a higher MOI of n212 did not lead to reactivation of and expression of VP16 from the quiescent KM110-R genome, as indicated by the lack of expression of the truncated form of VP16 (Figure 3.5.3A lane 4). This confirms that, even under conditions where productive infection of the SI virus occurs, the quiescent genome is not reactivated in the absence of ICP0. In contrast, following SI with KOS, the quiescent KM110-R genome was reactivated, as indicated by the production of the truncated form of VP16 (Figure 3.5.3A lane 6).

3.5.2.3 Production of infectious progeny

A progeny assay was used to test for the production of progeny KM110-R and SI viruses (Figure 3.5.2). Samples were harvested for virus progeny, then titered on U20S cells in the presence of 3 mM HMBA. For quantification, plaques were counted two days later using either a light or a fluorescent microscope. Samples infected with a MOI of 10 of KOS or MOI of 30 of n212 were harvested immediately following the Acid/Glycine wash in order to determine if unabsorbed virions would affect our results. We found that $2.24 \times 10^4 \pm 1.10 \times 10^4$ PFU/mL KOS and $3.69 \times 10^4 \pm 8.22 \times 10^3$ PFU/mL n212 remained following the Acid/Glycine wash. These values are approximately 1000 times lower than the number of KOS and n212 progeny virus resulting following productive infection, and therefore do not affect our results. As previously described, progeny virus produced from the reactivated quiescent KM110-R genome can be distinguished from progeny virus produced from the SI virus by the production of DsRed2 from the KM110-R progeny. The value reported for each sample includes the average of four independent experiments and the standard deviation.

Following n212 SI, we found that the number of n212 progeny produced was $3.66 \times 10^7 \pm 1.3 \times 10^7$ PFU/mL on mock treated cells and $1.79 \times 10^7 \pm 8.6 \times 10^6$ PFU/mL on KM110-R treated cells (Figure 3.5.4B n212). This may indicate there is some inhibition of the production of n212 progeny in KM110-R infected cells. We found that the production of KOS progeny following SI with KOS was inhibited to some extent on cells that had previously been infected with KM110-R compared to cells that had been mock treated. The amount KOS progeny produced on KM110-R infected cells was 2.63×10^7

± 3.9 x 10^6 PFU/mL compared to 5.52 x 10^7 ± 2.2 x 10^7 PFU/mL on mock treated cells (Figure 3.5.4B KOS). SI with n212 lead to the production of far less progeny KM110-R than did SI with KOS. SI of KM110-R infected cells with n212 led to the production of 1.00 x 10^4 ± 6.8 x 10^3 PFU/mL KM110-R (Figure 3.5.4C n212), a number similar to the 1.25 x 10^3 ± 1.5 x 10^3 PFU/mL resulting from mock treatment of KM110-R infected cells (Figure 3.5.4C mock). This confirms that, even under conditions where productive infection of the SI virus occurs, the quiescent genome is not reactivated in the absence of ICP0. In contrast, following SI with KOS, the quiescent KM110-R genome was reactivated, as indicated by the production of 8.30 x 10^6 ± 7.4 x 10^5 PFU/mL progeny KM110-R (Figure 3.5.4C KOS).

3.5.3 Summary

The results of these indirect IFA, Southern, Western and progeny assay experiments have shown that, in the absence of ICP0, the quiescent KM110-R genome is silent in the presence of an almost identical, replicating virus. This indicates the quiescent genome has been made inaccessible to transcription and DNA replication machinery. Possible locations and structures responsible for the silencing of the quiescent genome are of interest for further study, and will be revisited in the discussion.

3.6 FACS analysis of indirect IFA for U_S11 or gC leads to an overestimate of the proportion of productively infected cells

Another approach for determining the proportion of productively infected cells is to use FACS analysis following indirect IFA of late viral gene products. U_S11 and gC were chosen for this purpose because they are both true late proteins that are expressed only following replication of viral DNA

(Roizman & Knipe, 2001), and so would be good indicators for completion of the lytic cycle. We first tried FACS analysis of indirect IFA using a primary Ab for U_S11, and an Alexa Fluor 488 (green fluorescent) secondary Ab. HEL cells were mock treated or infected with a MOI of 10 of KOS. After 18 hrs the cells were harvested and prepared for indirect IFA using the protocol for cells in suspension (Materials and Methods 2.8.2) and analyzed by FACS. The results are presented as histograms with increasing counts shown on the y-axis, and increasing green fluorescence shown on the x-axis. The preliminary results seemed promising as uninfected cells (Mock) had very low background fluorescence (Figure 3.6.1A left) while infected cells (KOS) had a strong fluorescent signal (Figure 3.6.1A right), and there was no overlap between the negative (uninfected) and positive (infected) cell signals. However, as a control, a mixture of half mock and half KOS treated cells was combined just after the 18 hr infection period, but before treatment for indirect IFA. This control was important because, in the experimental system this analysis would be used for, the samples harvested would be expected to include both infected and uninfected cells. FACS analysis revealed that mixing infected and uninfected cells did not result in the expected distinct populations of positive and negatively staining cells. Instead, more than half the cells stained positive while the rest stained a slightly lower fluorescence than positive, but higher than expected for negative (Figure 3.6.1B). This result indicates that U_S11 leaks from infected cells to uninfected cells during preparation of samples for FACS analysis of indirect IFA. The tendency of U_S11 to leak out of infected cells may be due to its small (17, 000 kDa) size.

Therefore, we next tried indirect IFA using a primary Ab for the viral late protein gC, which has a MW of 100, 000 kDa, and an Alexa Fluor 488 secondary Ab. HEL cells were mock treated, or infected with a MOI of 10 of KOS. After 18 hrs the cells were harvested and prepared for indirect IFA using the protocol for cells in suspension (Materials and Methods 2.8.2) and analyzed by FACS. The results are presented as histograms with increasing counts shown on the y-axis, and increasing green fluorescence shown on the x-axis. Again, the preliminary results seemed promising with low background fluorescence of uninfected cells (Figure 3.6.2A left), high fluorescence of infected cells (Figure 3.6.2A right), and no overlap between the negative and positive cell signals. Additionally, a mixture of half mock and half KOS treated cells combined before treatment for indirect IFA did not lead to the problem of gC leaking in to uninfected cells that was previously seen for U_S11. The expected proportion of negative to positively staining cells was seen, although the fluorescence signal for uninfected cells was slightly increased under these conditions (Figure 3.6.2B). However, because gC is a virion envelope associated protein, we tested to see if virion associated gC attaching to the cell could lead to a positive signal in the absence of productive infection. HEL cells were infected with a MOI of 100 of KOS in the presence or absence of 400 µg/ml PAA. An MOI of 100 was chosen because we estimated this to be reasonably close to the number of virus particles a cell would be exposed to following the release of progeny virus from nearby, infected cells. PAA was used in order to prevent DNA replication, and therefore production of gC. After 18 hrs the cells were harvested, prepared for indirect IFA using the protocol for cells in suspension (Materials and Methods 2.8.2), and analyzed by FACS. Our results show that, even under conditions where no gC is produced (Figure 3.6.2C Plus PAA), the fluorescent signal was the same as for conditions where gC was produced (Figure 3.6.2C No PAA). This indicates that at a MOI of 100 of KOS, enough gC comes in with the virion to give the cells a positive signal. Therefore, FACS analysis of indirect IFA using primary Abs to U_S11 and gC were decided to be unsuitable for determining the proportion of infected cells.

Figure 3.1.1: Structure of the plasmids used to construct fluorescent recombinant HSV-1 strains.

PTK-Red and pTK-Green consist of the background vector pTK 173 and an insert fragment from pDsRed2-C1 or pEGFP-C1 respectively. The pTK 173 vector includes the HSV-1 thymidine kinase (TK) locus which has a unique Sst I (S) site. The insert fragment was cloned into the Sst I site (destroying this site) and includes a Human cytomegalovirus immediate early (CMV IE) promoter, the fluorescent gene *Discosoma sp.* Human codon-optimized Red Fluorescent protein (DsRed2) or enhanced green fluorescent protein (EGFP), a multiple cloning site (MCS), and a Simian virus 40 early mRNA polyadenylation (SV40 Poly A) signal. See Materials and Methods for a more detailed description of the construction of these plasmids.

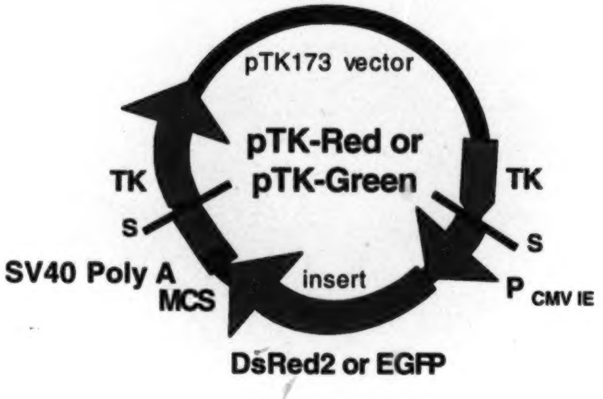

pTK173 vector
pTK-Red or pTK-Green
TK
TK
S
S
SV40 Poly A
MCS
insert
P CMV IE
DsRed2 or EGFP

Figure 3.1.2: Structure of fluorescent recombinant HSV-1 strains. DsRed2 or EGFP, under the control of the CMV IE promoter, were inserted into the thymidine kinase locus of HSV-1 strains KOS, n212, and KM110 by homologous recombination with pTK-Red or pTK-Green. (A-C) Schematic representations of the genomes of KOS-Green (KOS-G), n212-Green (n212-G), and KM110-Red (KM110-R). The HSV-1 genome is divided into unique long (U_L) and unique short (U_S) each flanked by terminal repeats (TR_L or TR_S) and internal repeats (IR_L or IR_S). The mutations made to each strain are shown in expansions of the affected loci.

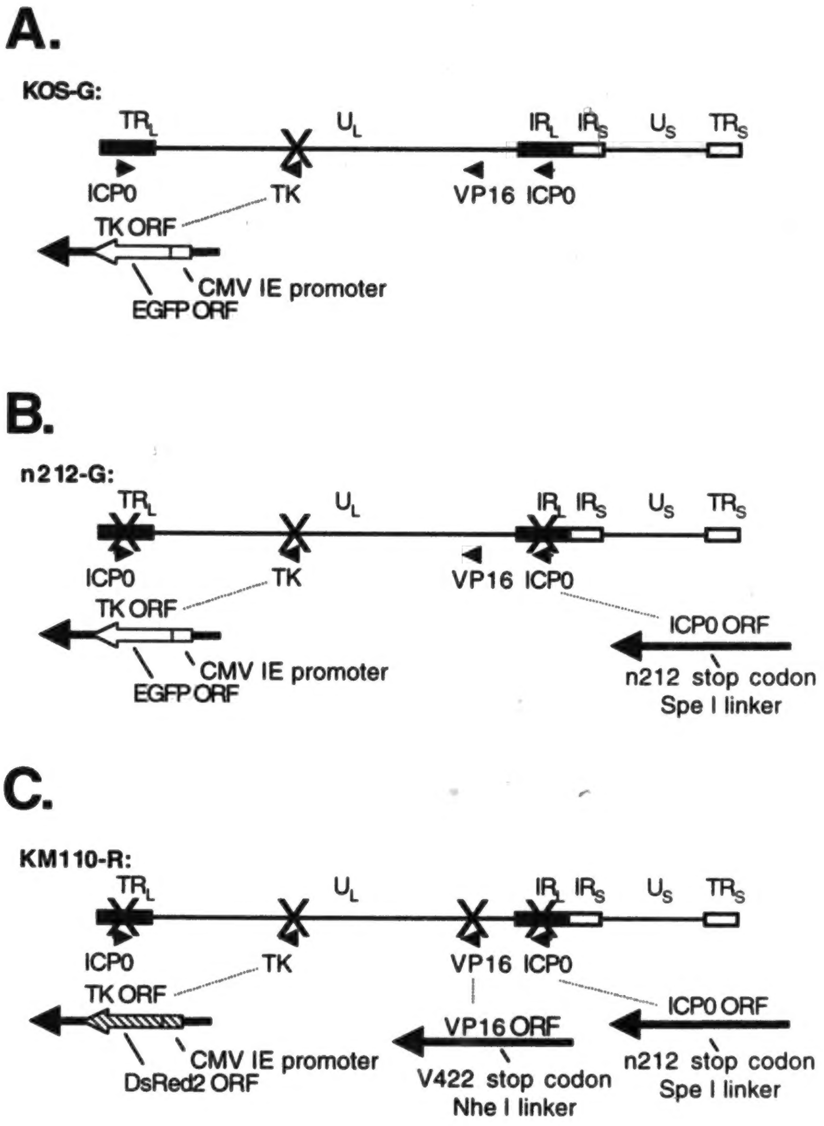

A.
KOS-G:
TR$_L$
U$_L$
IR$_L$
IR$_S$
U$_S$
TR$_S$
ICP0
TK
VP16
ICP0
TK ORF
CMV IE promoter
EGFP ORF
B.
n212-G:
TR$_L$
U$_L$
IR$_L$
IR$_S$
U$_S$
TR$_S$
ICP0
TK
VP16
ICP0
TK ORF
ICP0 ORF
CMV IE promoter
EGFP ORF
n212 stop codon
Spe I linker
C.
KM110-R:
TR$_L$
U$_L$
IR$_L$
IR$_S$
U$_S$
TR$_S$
ICP0
TK
VP16
ICP0
TK ORF
ICP0 ORF
VP16 ORF
CMV IE promoter
DsRed2 ORF
V422 stop codon
Nhe I linker
n212 stop codon
Spe I linker

Figure 3.1.3: Diagram of the restriction map of the DNA from wt and recombinant strains.

The open reading frame (ORF), region of probe hybridization, restriction sites (*italics*) and expected fragment sizes (kbp) of each of the indicated strains are shown for the VP16 locus (A), the ICP0 locus (B), and the TK locus (C).

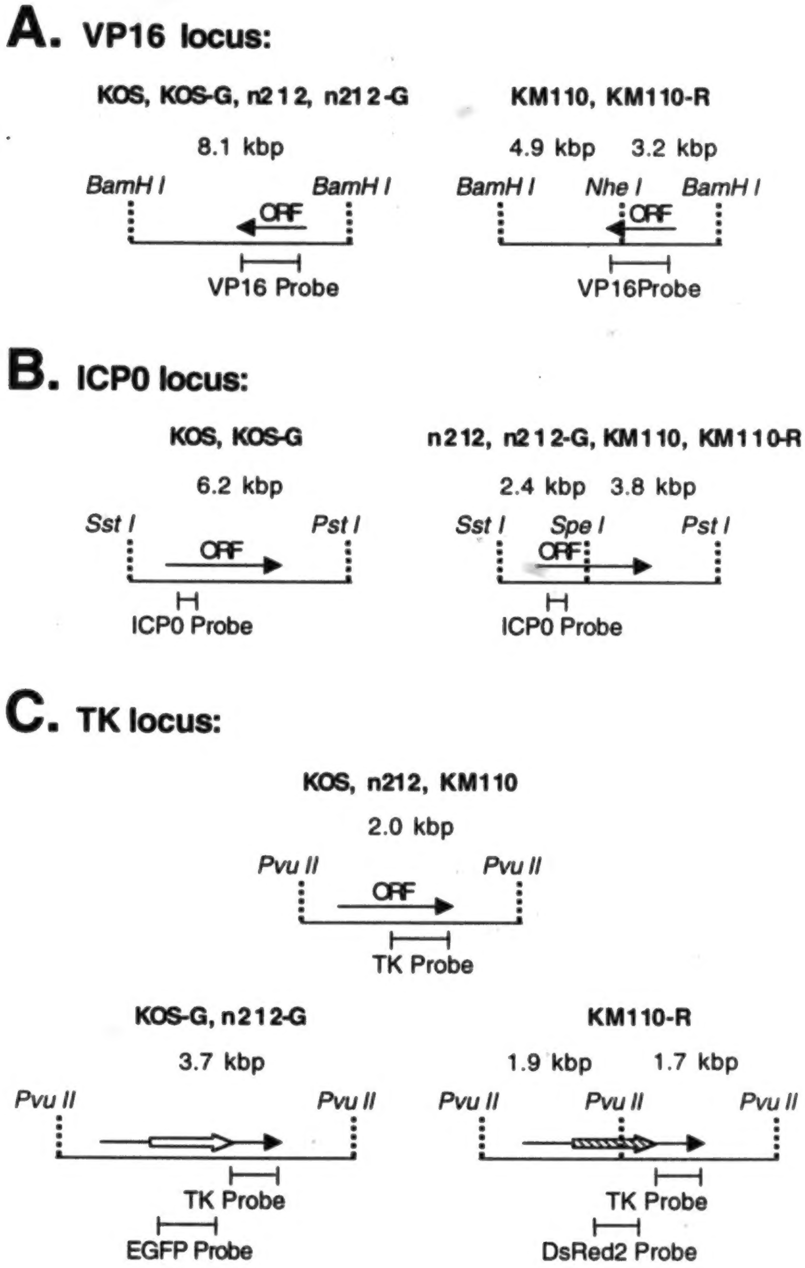

A. VP16 locus:
KOS, KOS-G, n212, n212-G
8.1 kbp
BamH I
BamH I
ORF
VP16 Probe
KM110, KM110-R
4.9 kbp
3.2 kbp
BamH I
Nhe I
BamH I
ORF
VP16Probe
B. ICP0 locus:
KOS, KOS-G
6.2 kbp
Sst I
Pst I
ORF
ICP0 Probe
n212, n212-G, KM110, KM110-R
2.4 kbp
3.8 kbp
Sst I
Spe I
Pst I
ORF
ICP0 Probe
C. TK locus:
KOS, n212, KM110
2.0 kbp
Pvu II
Pvu II
ORF
TK Probe
KOS-G, n212-G
3.7 kbp
Pvu II
Pvu II
TK Probe
EGFP Probe
KM110-R
1.9 kbp
1.7 kbp
Pvu II
Pvu II
Pvu II
TK Probe
DsRed2 Probe

Figure 3.1.4: Confirmation of the identity of wt and recombinant HSV-1 strains.

Southern blot analysis. Confluent monolayers of U2OS cells were infected with MOI 10 of the indicated virus. The samples were harvested after 24 hrs and prepared for Southern analysis. The DNA of the wt and recombinant strains was digested with BamH I/Nhe I (A), Sst I/Spe I/Pst I (B), or Pvu II (C-E), and hybridized with the indicated probe. The results confirm the presence of the Nhe I linker in KM110 and KM110-Red; the Spe I linker in n212, n212-Green, KM110, and KM110-Red; the EGFP insert in KOS-Green and n212-Green; and the DsRed2 insert in KM110-Red.

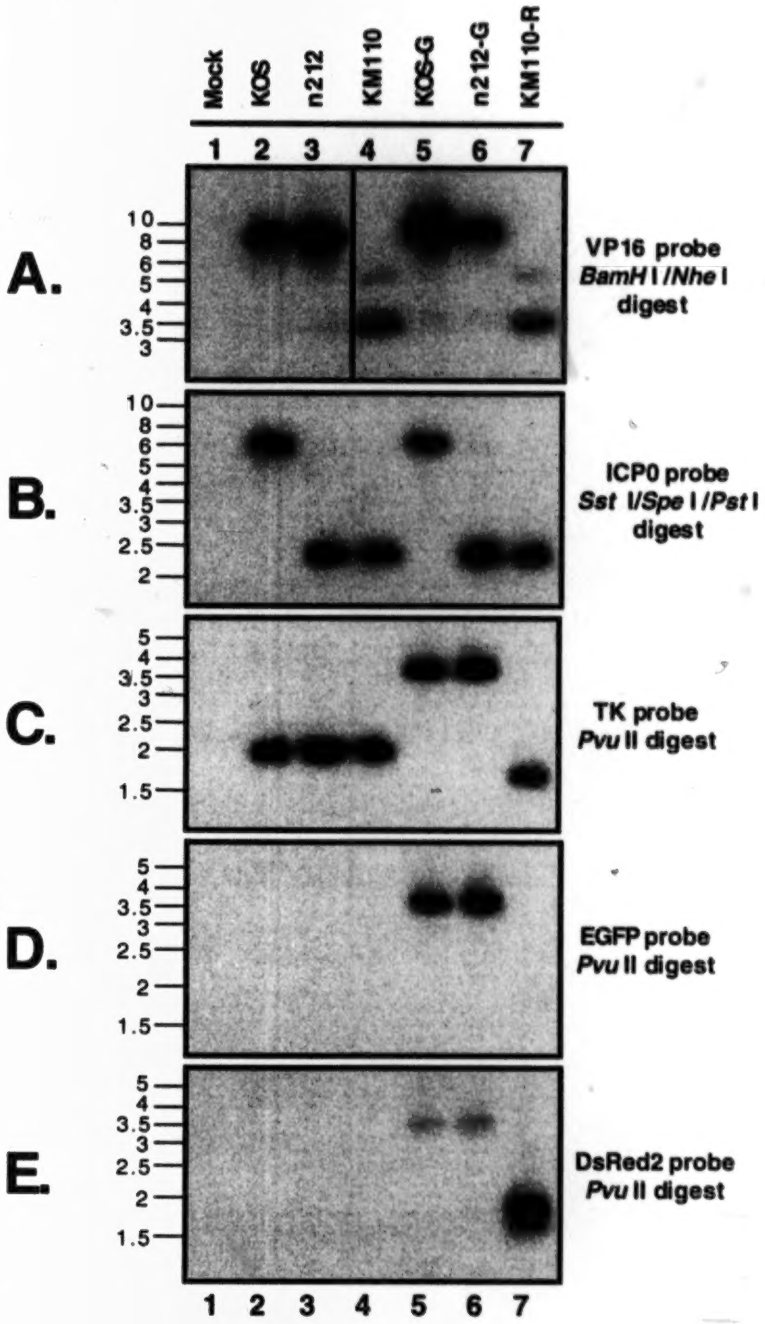

Mock
KOS
n212
KM110
KOS-G
n212-G
KM110-R
1 2 3 4 5 6 7
A.
10
8
6
5
4
3.5
3
VP16 probe
BamH I /*Nhe* I
digest
B.
10
8
6
5
4
3.5
3
2.5
2
ICP0 probe
Sst I/*Spe* I /*Pst* I
digest
C.
5
4
3.5
3
2.5
2
1.5
TK probe
Pvu II digest
D.
5
4
3.5
3
2.5
2
1.5
EGFP probe
Pvu II digest
E.
5
4
3.5
3
2.5
2
1.5
DsRed2 probe
Pvu II digest
1 2 3 4 5 6 7

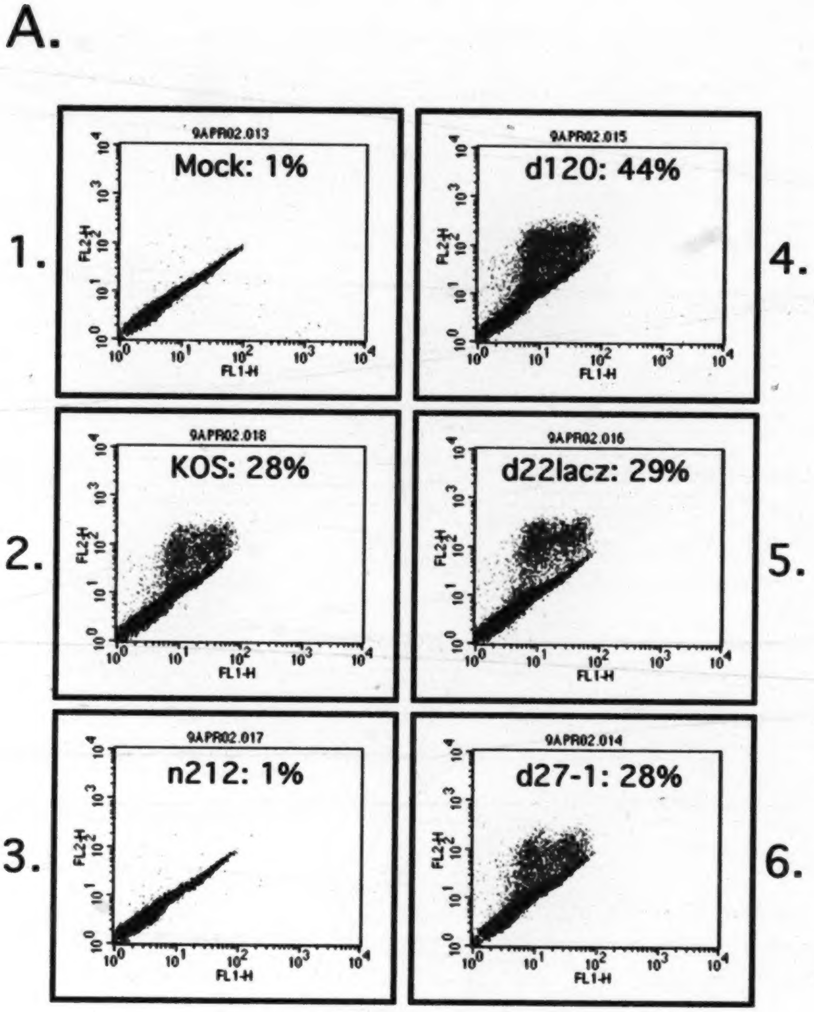
Figure 3.1.5: The fluorescent gene from the recombinant virus is expressed during productive infection.

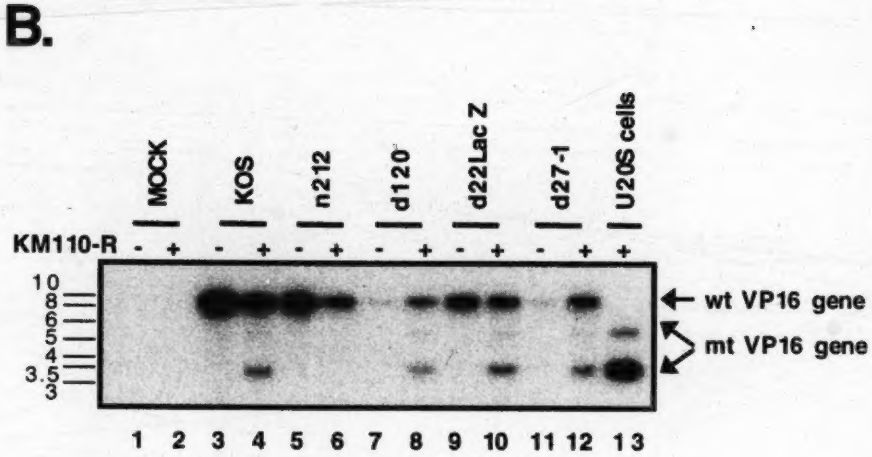
Fluorescent microscope photographs of n212-Green (A) and KM110-Red (B) plaques on U2OS cells. Both viruses were plated at a low MOI (with HMBA added to the infecting media of KM110-Red) to give rise to individual plaques. The plates were photographed two days post-viral infection.

A.

B.

Figure 3.2: ICP0 is a specific requirement for the reactivation of DNA replication and gene expression of quiescent HSV-1. Confluent monolayers of HEL cells were infected with KM110-Red MOI 2 (A) or MOI 6 (B and C) in order to establish a quiescent infection. Four days later the cells were superinfected (SI) with wt or an immediate early (IE) mutant at an MOI of 10. The next day the samples were harvested and analyzed by (A) FACS showing the intensity of red fluorescence on the y-axis and green fluorescence on the x-axis; (B) Southern with a BamH I/Nhe I restriction digest and VP16 probe; or (C) Western using Ab to actin, VP16, and U_S11. The percent of cells expressing red fluorescence is specified for each sample. The size of the markers is shown on the left in kDa (A) or kbp (B).


A.
1.
2.
3.
4.
5.
6.
9APR02.013
Mock: 1%
9APR02.018
KOS: 28%
9APR02.017
n212: 1%
9APR02.015
d120: 44%
9APR02.016
d22lacz: 29%
9APR02.014
d27-1: 28%
FL2-H
FL1-H
10^0
10^1
10^2
10^3
10^4


B.
MOCK
KOS
n212
d120
d22Lac Z
d27-1
U20S cells
KM110-R
- + - + - + - + - + - + +
10
8
6
5
4
3.5
3
wt VP16 gene
mt VP16 gene
1 2 3 4 5 6 7 8 9 10 11 12 13

C.

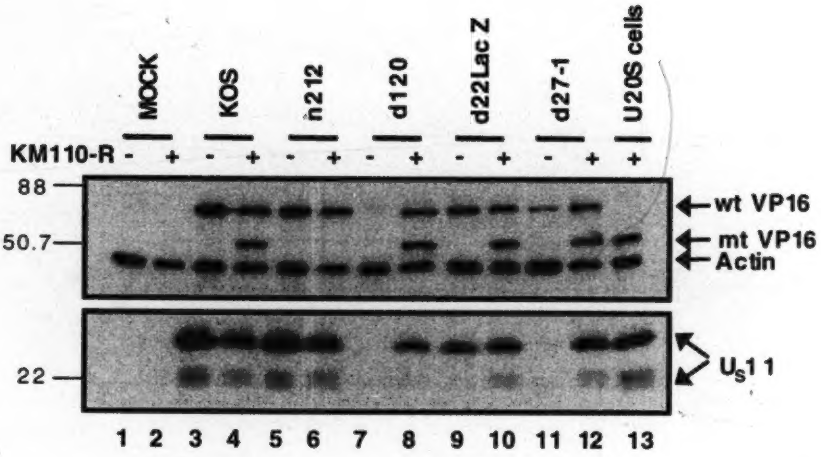
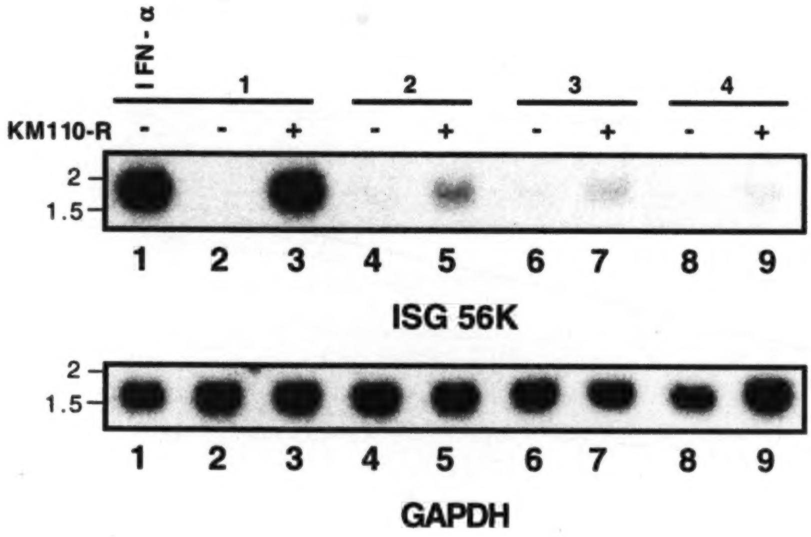

MOCK
KOS
n212
d120
d22Lac Z
d27-1
U20S cells
KM110-R - + - + - + - + - + - + +
88
50.7
22
wt VP16
mt VP16
Actin
U_S11
1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 3.3.1: The antiviral response induced by KM110-Red dissipates by four days post-infection.

(A) Northern blot analysis of the antiviral response using a probe for ISG-56K (Top) or GAPDH (Bottom). Confluent monolayers of HEL cells were treated with 1000 U/mL IFN-α, mock treated (-) or infected with KM110-Red MOI 6 (+). The samples were harvested the indicated number of days post-treatment and analyzed by northern blot. The size of the RNA markers in kbp is shown on the left. (B) Quantification of northern blot analysis. The quantity of ISG-56K mRNA was determined using phosphor-imager analysis, normalized to GAPDH, and plotted against the indicated time points. The results of two independent experiments are shown.

A.


IFN - α
1
2
3
4
KM110-R
- - + - + - + - +
2
1.5
1 2 3 4 5 6 7 8 9
ISG 56K
2
1.5
1 2 3 4 5 6 7 8 9
GAPDH

B.

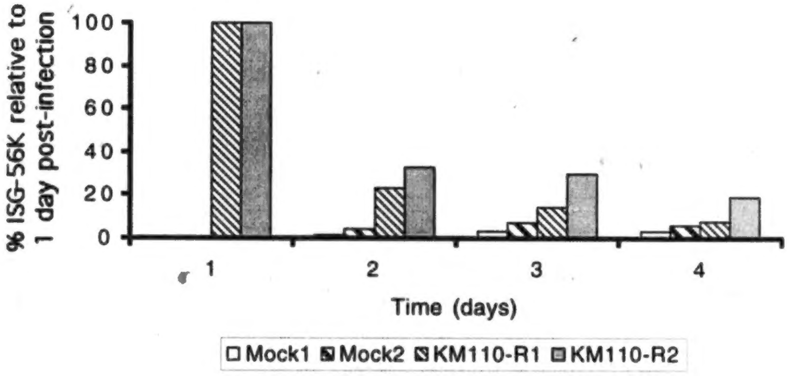

% ISG-56K relative to 1 day post-infection
100
80
60
40
20
0
1
2
3
4
Time (days)
□ Mock1 ▨ Mock2 ▨ KM110-R1 ▨ KM110-R2

Figure 3.3.2: The antiviral response induced by KM110-Red inhibits KOS to a lesser extent 4 days post-induction than it does 1 day post-induction.

Plaque assay. Confluent monolayers of HEL cells were either mock treated or infected with KM110-Red MOI 6. After 1 day (A) or 4 days (B) the samples were either mock treated or SI with 2.7 x 10^3 PFU KOS. The media was replaced with 0.5% methylcellulose /10%FBS/DMEM and the plates were incubated for 5 days before being Giemsa stained. These results were consistent for two independent experiments.

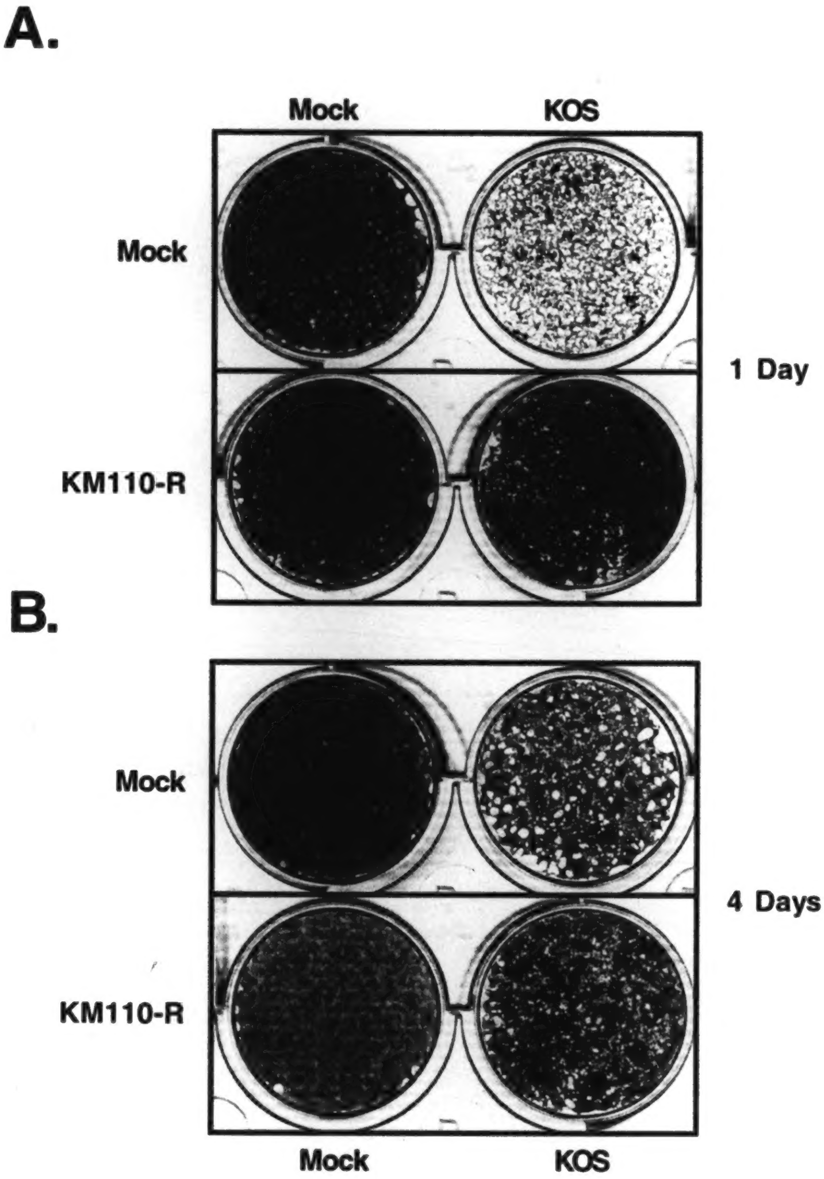

A.
Mock
KOS
Mock
KM110-R
1 Day
B.
Mock
KM110-R
4 Days
Mock
KOS

Figure 3.4.1: ICP0 mutants are able to superinfect HEL cells harbouring the quiescent KM110-Red genome.

FACS analysis. (A-E) Confluent monolayers of HEL cells were infected with KM110-Red MOI 6. 4 days later the cells were mock treated or SI with MOI 30 of n212 or n212-green, or MOI 10 of KOS or KOS-Green. 18 hrs post-SI the samples were harvested and analyzed by FACS. The intensity of red fluorescence is shown on the y-axis, and green fluorescence on the x-axis. The red and green dots indicate cells expressing red and green fluorescence respectively. The purple dots indicate cells expressing both red and green fluorescence. The % of cells expressing fluorescence is specified for each sample. The standard deviation was calculated from four independent experiments.

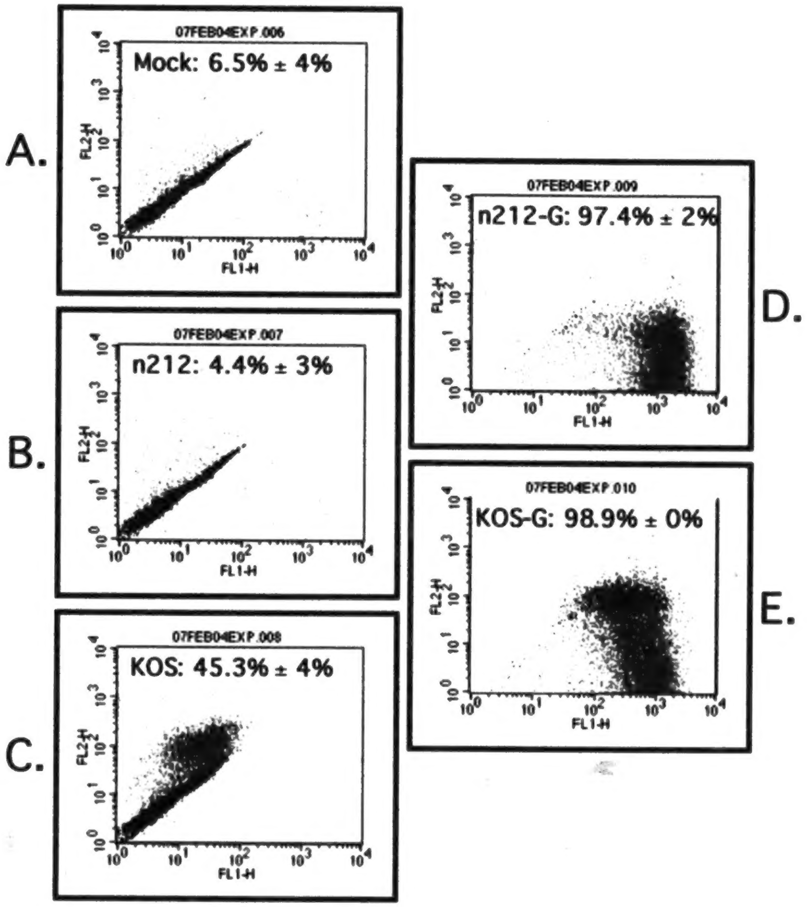
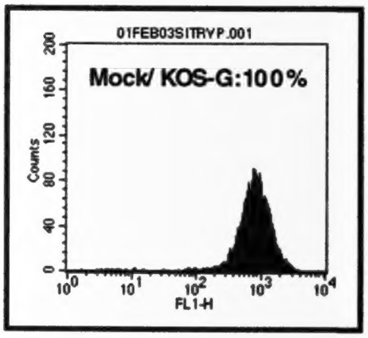

A.
07FEB04EXP.006
Mock: 6.5% ± 4%
B.
07FEB04EXP.007
n212: 4.4% ± 3%
C.
07FEB04EXP.008
KOS: 45.3% ± 4%
D.
07FEB04EXP.009
n212-G: 97.4% ± 2%
E.
07FEB04EXP.010
KOS-G: 98.9% ± 0%
FL2-H
FL1-H
10⁰
10¹
10²
10³
10⁴

Figure 3.4.2: Validation of using reactivation of KM110-Red by KOS for the quantification of cells harbouring the quiescent genome.

FACS analysis of the ability of HSV-1 to infect cells productively infected with HSV-1. Confluent monolayers of HEL cells were mock treated or infected with KOS MOI 10. After 12 hrs, the cells were SI with KOS-Green MOI 10. Fifteen hrs post-SI (27 hrs PI) the samples were harvested and analyzed by FACS. The number of counts is shown on the y-axis and intensity of green fluorescence is shown on the x-axis. The percent of cells expressing green fluorescence is indicated for each sample. These results were consistent for 2 independent experiments.


01FEB03SITRYP.001
Mock/ KOS-G:100%
Counts
0
40
80
120
160
200
10^0
10^1
10^2
10^3
10^4
FL1-H

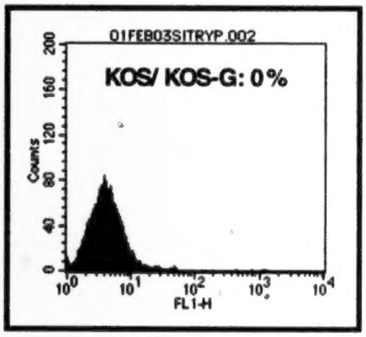
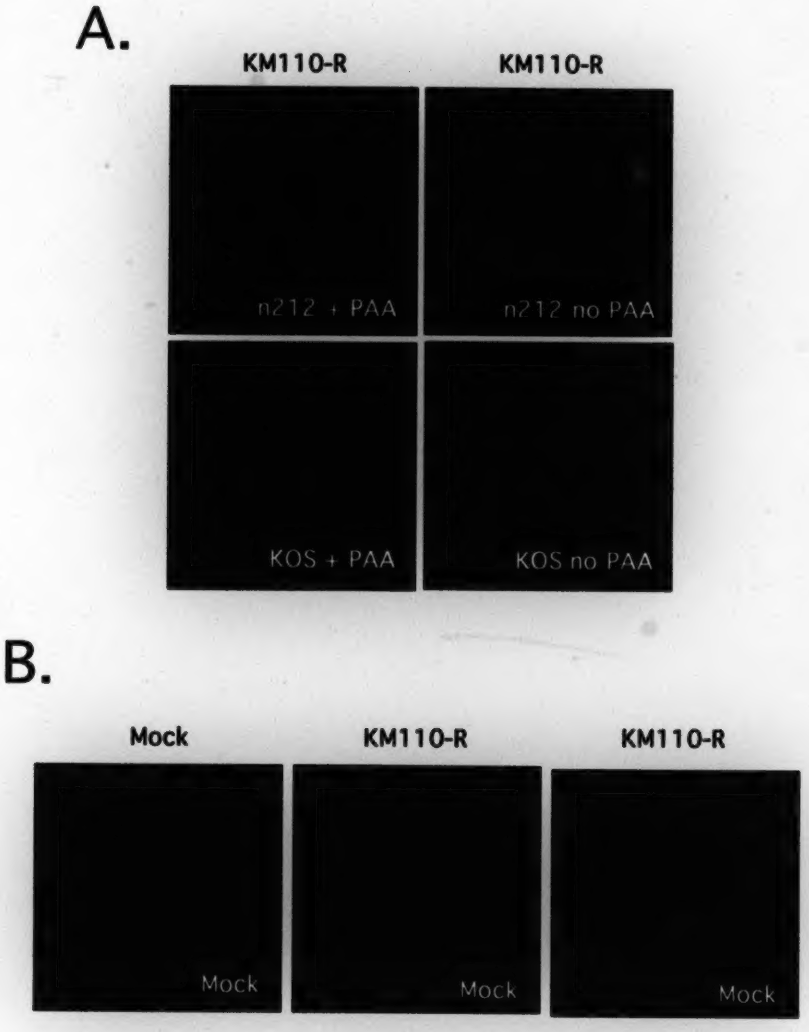
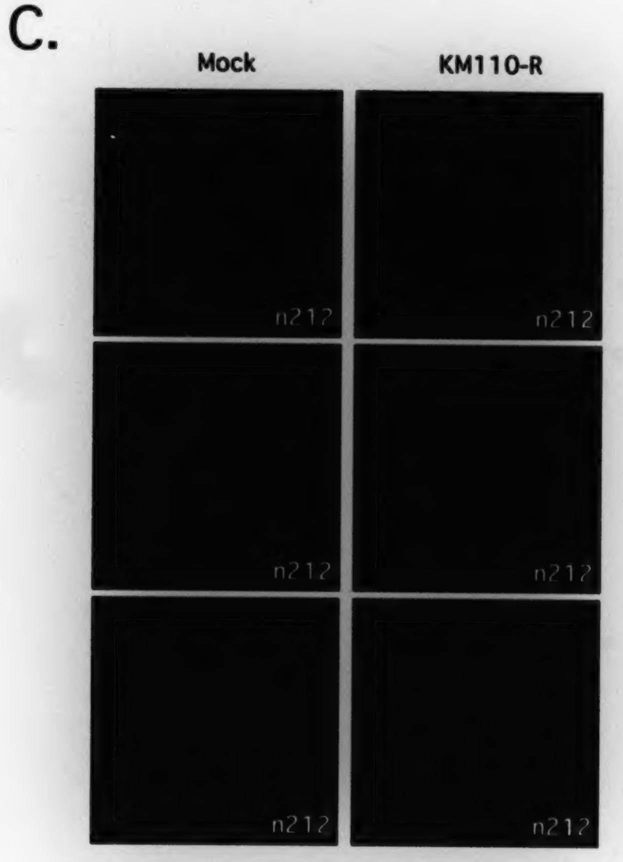
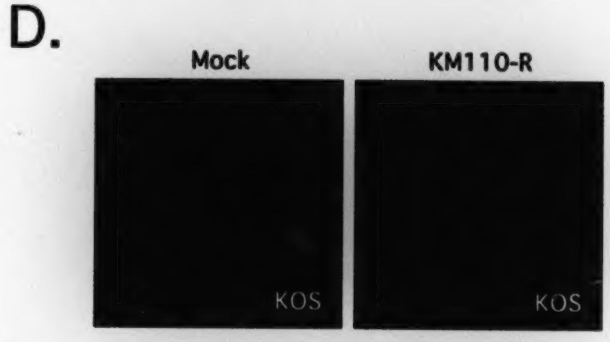

01FEB03SITRYP.002
KOS/ KOS-G: 0%
Counts
0
40
80
120
160
200
10^0
10^1
10^2
10^3
10^4
FL1-H

Figure 3.5.1: n212 is able to form replication compartments in cells harbouring the quiescent KM110-Red genome.

(A-D) Confocal microscope pictures of indirect IFA for replication compartments. Confluent monolayers of HEL cells grown on coverslips were mock treated or infected with KM110-Red MOI 6. 4 days post-infection the cells were: (A) SI with n212 MOI 30 or KOS MOI 10 in the presence or absence of 400 µg/mL PAA (pictures shown for no PAA are the same as for C and D); (B) mock treated; (C) SI with n212 MOI 30; or (D) SI with KOS MOI 10. 9 1/2 hrs post-SI the samples were fixed and treated for indirect IFA of cell monolayers (Materials and Methods 2.8.1) using a primary Ab to ICP4, an Alex Fluor secondary Ab, and Hoescht nuclei stain. The pictures were taken by a confocal microscope under a 40X oil objective lens. (E) Quantification of indirect IFA assay. Fluorescent microscope pictures were taken of each sample. From these, 100-650 cells were scored as either positive or negative for replication compartments. The bars represent the standard deviation over three independent experiments.


A.
KM110-R
KM110-R
n212 + PAA
n212 no PAA
KOS + PAA
KOS no PAA
B.
Mock
KM110-R
KM110-R
Mock
Mock
Mock


C.
Mock
KM110-R
n212
n212
n212
n212
n212
n212


D.
Mock
KM110-R
KOS
KOS

E.

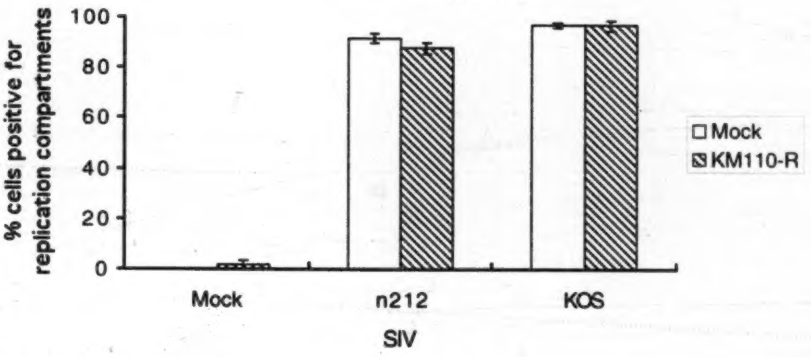
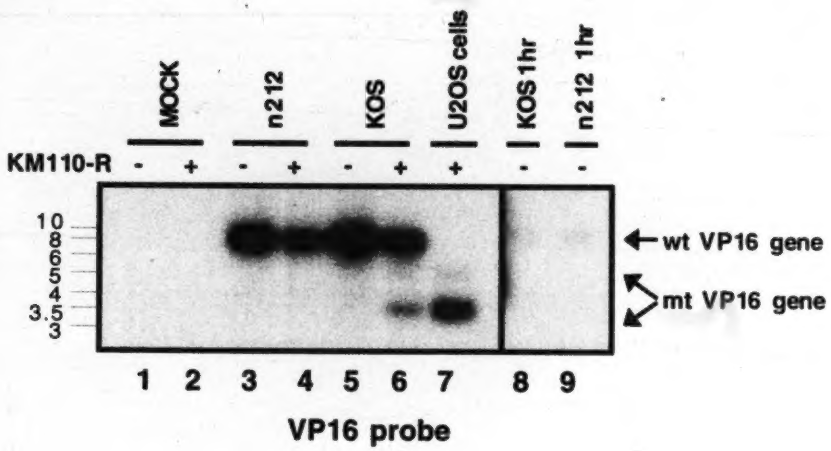

% cells positive for replication compartments
100
80
60
40
20
0
Mock
n212
KOS
SIV
□ Mock
▨ KM110-R

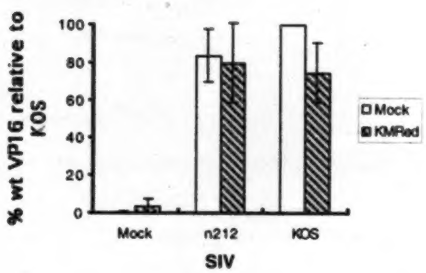
Figure 3.5.2: n212 is able to replicate its own genome in cells harbouring the quiescent KM110-Red genome, but is unable to reactivate replication from the quiescent KM110-Red genome.

(A) Southern Assay. Confluent monolayers of HEL cells were mock treated or infected with KM110-Red MOI 6. 4 days post-infection the cells were mock treated or SI with n212 MOI 30 or KOS MOI 10. Eighteen hrs post-SI the cells were harvested for Southern. The DNA was BamHI/NheI restriction digested, and the blot was hybridized to a VP16 probe. The size of the DNA markers is shown on the left in kbp. (B and C) Quantification of Southern blot analysis. The quantity of wt VP16 DNA (B) or mt VP16 DNA (C) was determined using phosphor-imager analysis. This value was normalized to the amount of total DNA (B and C) and to the mt VP16 signal from the samples with no KM110 (C only). The error bars indicate the standard deviation over four independent experiments.

A.


MOCK
n212
KOS
U2OS cells
KOS 1hr
n212 1hr
KM110-R
- + - + - + + - -
10
8
6
5
4
3.5
3
wt VP16 gene
mt VP16 gene
1 2 3 4 5 6 7 8 9
VP16 probe

B.


% wt VP16 relative to KOS
100
80
60
40
20
0
Mock
n212
KOS
SIV
Mock
KMRed

C.

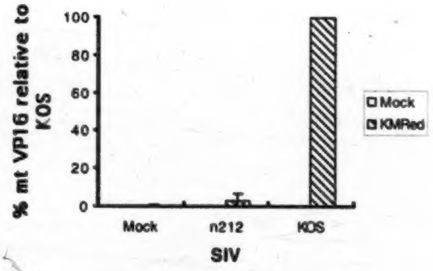

% mt VP16 relative to KOS
100
80
60
40
20
0
Mock
n212
KOS
SIV
Mock
KMRed

Figure 3.5.3: n212 is able to express late gene products from its own genome in cells harbouring the quiescent KM110-Red genome, but is unable to reactivate the production of late gene products from the quiescent KM110-Red.

(A) Western Assay. Confluent monolayers of HEL cells were mock treated or infected with KM110-Red MOI 6. 4 days post-infection the cells were mock treated or SI with n212 MOI 30 or KOS MOI 10. 18 hrs post-SI the cells were harvested for Western as described in Materials and Methods. The blot was incubated with monoclonal primary Abs to VP16, Actin, and U_S11. The size of the protein markers is shown on the left in kDa. (B-D) Quantification of Western blot analysis. The quantity of wt VP16 (B), mt VP16 (C), and U_S11 (D) was determined using phosphor-imager analysis. This value was normalized to the amount Actin in each sample (B-D) and to the mt VP16 signal from the samples with no KM110 (C only). The error bars indicate the standard deviation over four independent experiments.

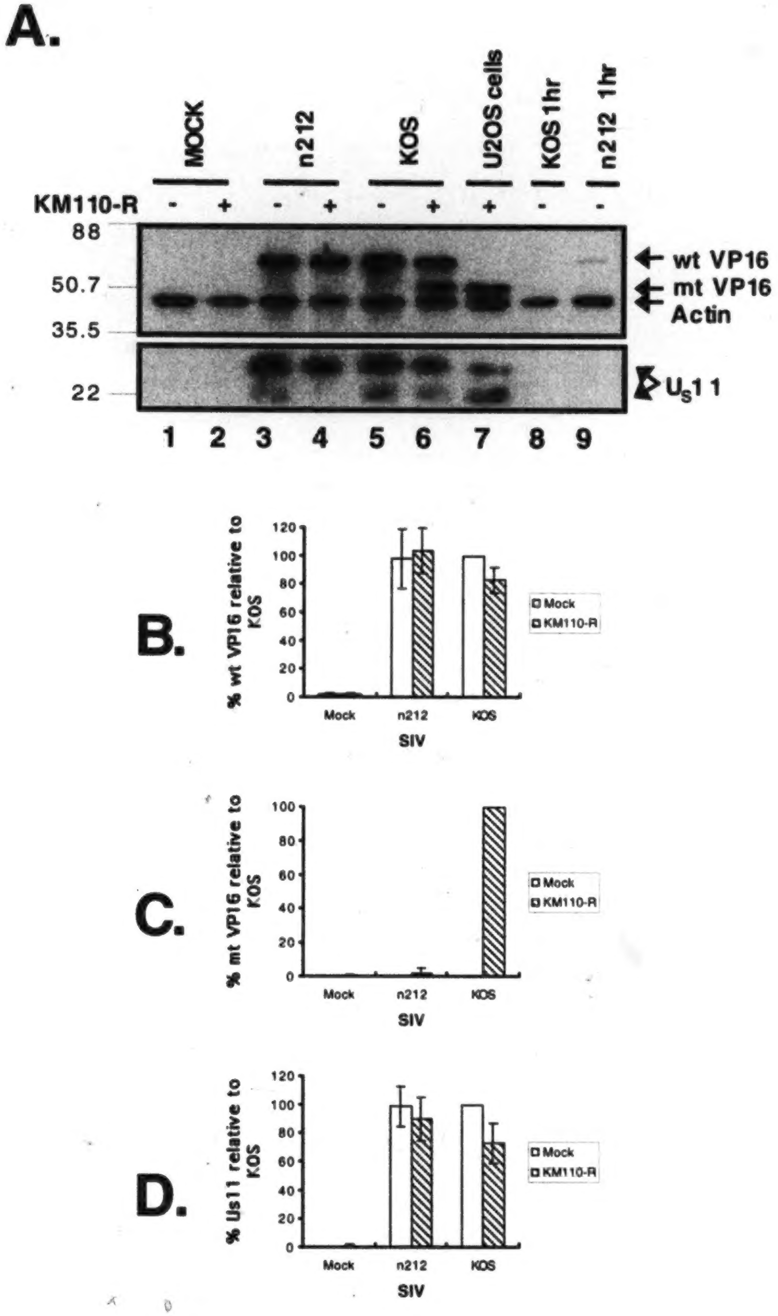

A.
MOCK
n212
KOS
U2OS cells
KOS 1hr
n212 1hr
KM110-R
- + - + - + + - -
88
50.7
35.5
22
wt VP16
mt VP16
Actin
U_S11
1 2 3 4 5 6 7 8 9
B.
% wt VP16 relative to KOS
120
100
80
60
40
20
0
Mock
n212
KOS
SIV
Mock
KM110-R
C.
% mt VP16 relative to KOS
100
80
60
40
20
0
Mock
n212
KOS
SIV
Mock
KM110-R
D.
% Us11 relative to KOS
120
100
80
60
40
20
0
Mock
n212
KOS
SIV
Mock
KM110-R

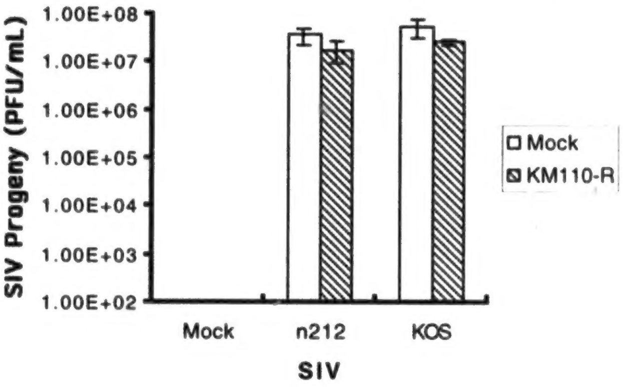
Figure 3.5.4: n212 is able to produce progeny from its own genome in cells harbouring the quiescent KM110-Red genome, but is unable to reactivate the production of progeny from the quiescent KM110-Red genome.

Progeny Assay. Confluent monolayers of HEL cells were mock treated or infected with KM110-Red MOI 6. 4 days post-infection the cells were mock treated or SI with n212 MOI 30 or KOS MOI 10. 2 hrs post-SI the cells were Acid Glycine Washed to remove unabsorbed virus. 18 hrs post-SI the cells were harvested for virus progeny as described in Materials and Methods 2.3. The samples were titered on U20S cells in the presence of 3 mM HMBA and the plaques were counted 2 days later using either a light or a fluorescent microscope. A table (A) and graphs (B and C) summarize the resulting viral titers. The standard deviation, represented as ± (A) or bars (B and C) was calculated from four independent experiments.

A.

Superinfecting Virus (SIV)	KM110-R (+ or -)	SIV Titer (PFU/mL)	KM110-R Titer (PFU/mL)
Mock	-	0	N/A
	+	0	1.25×10^3 $\pm 1.5 \times 10^3$
n212	-	3.66×10^7 $\pm 1.3 \times 10^7$	N/A
	+	1.79×10^7 $\pm 8.6 \times 10^6$	1.00×10^4 $\pm 6.8 \times 10^3$
KOS	-	5.52×10^7 $\pm 2.2 \times 10^7$	N/A
	+	2.63×10^7 $\pm 3.9 \times 10^6$	8.30×10^6 $\pm 7.4 \times 10^5$

B.


SIV Progeny (PFU/mL)
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
Mock
n212
KOS
SIV
□ Mock
▨ KM110-R

C.

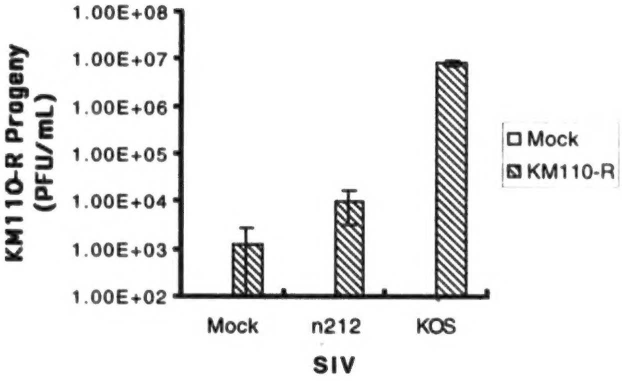

KM110-R Progeny (PFU/mL)
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
Mock
n212
KOS
SIV
□ Mock
▨ KM110-R

Figure 3.6.1: U_S11 leaks from infected cells to uninfected cells during preparation of samples for FACS analysis of indirect IFA. Confluent monolayers of HEL cells were mock treated, or infected with KOS MOI 10. After 18 hrs the cells were harvested and prepared for indirect IFA using the protocol for cells in suspension (Materials and Methods 2.8.2) and analyzed by FACS. A monoclonal primary Ab to U_S11 and an Alexa-fluor goat α-mouse secondary Ab were used. The number of counts is shown on the y-axis, and the intensity of green fluorescence is shown on the x-axis. (A) Mock treated or KOS infected cells. (B) A mixture of 1/2 Mock treated and 1/2 KOS infected cells (cells were mixed together after the 18 hr infection period, but before treatment for indirect IFA).

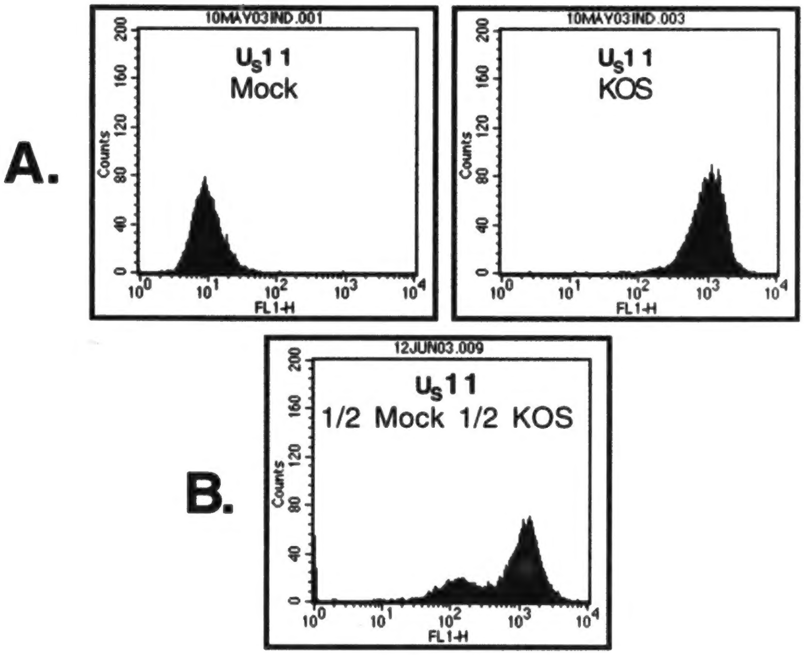
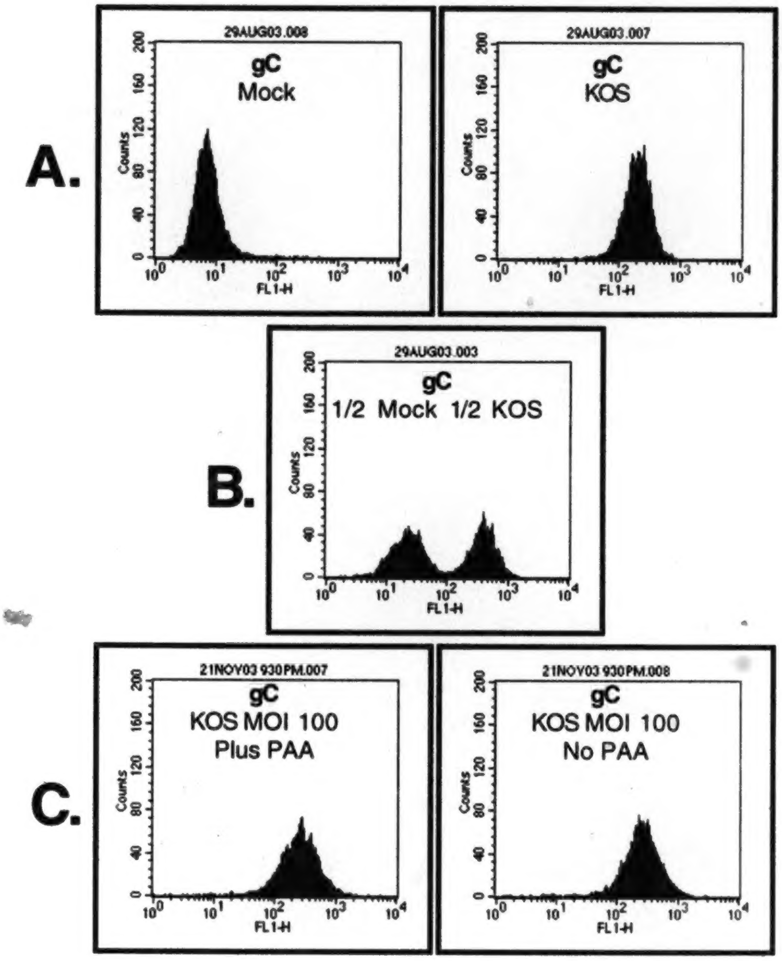

A.
10MAY03IND.001
U_S11
Mock
10MAY03IND.003
U_S11
KOS
B.
12JUN03.009
U_S11
1/2 Mock 1/2 KOS
Counts
0
40
80
120
160
200
10^0
10^1
10^2
10^3
10^4
FL1-H

Figure 3.6.2: At high MOI, enough gC comes in with the virion to give a positive signal for FACS analysis of indirect IFA.

Confluent monolayers of HEL cells were mock treated, or infected with KOS MOI 10 or 100. After 18 hrs the cells were harvested and prepared for indirect IFA using the protocol for cells in suspension (Materials and Methods 2.8.2) and analyzed by FACS. A monoclonal gC Ab was used for the primary Ab and Alexa-fluor goat α-mouse Ab was used for the secondary Ab. The number of counts is shown on the y-axis, and the intensity of green fluorescence is shown on the x-axis. (A) Mock treated or KOS MOI 10 infected cells. (B) A mixture of 1/2 Mock treated and 1/2 KOS MOI 10 infected cells (cells were mixed together after the 18 hr infection period, but before treatment for indirect IFA). (C) Cells infected with MOI 100 KOS in the presence or absence of 400 μg/ml PAA.


A.
29AUG03.008
gC
Mock
29AUG03.007
gC
KOS
B.
29AUG03.003
gC
1/2 Mock 1/2 KOS
C.
21NOV03 930PM.007
gC
KOS MOI 100
Plus PAA
21NOV03 930PM.008
gC
KOS MOI 100
No PAA
Counts
0
40
80
120
160
200
10^0
10^1
10^2
10^3
10^4
FL1-H

CHAPTER 4 : DISCUSSION

4.1 Summary of thesis results:

The experiments described in this investigation were done in order to determine if the nature of the repression of a quiescent mutant virus is such that a quiescent viral genome will remain silent even in the presence of an almost identical, replicating virus. HSV mutants that are unable to express E genes become quiescent following infection (Roizman & Knipe, 2001). Once this state has become established, no viral gene products are produced and the viral genome can be reactivated only by the provision of ICP0, its functional homologues from other herpesviruses, or the histone deacetylase (HDAC) inhibitor trichostatin A (TSA) (Everett *et al.*, 1998b, Harris & Preston, 1991, Hobbs & DeLuca, 1999, Preston & Nicholl, 1997, Samaniego *et al.*, 1998, Stow & Stow, 1989).

In order to confirm that ICP0 is necessary for the reactivation of a quiescent genome, and rule out the possibility that other HSV products are also required, we tested the ability of various HSV IE mutants to reactivate quiescent KM110-R. The results of FACS, Southern, and Western analysis all indicated that ICP0 is the only HSV product necessary for the reactivation of a quiescent genome. However, we also found that replication of the ICP0 mutant virus is inhibited on monolayers that had previously been infected with KM110-R. We therefore tested if a lingering antiviral response, triggered in response to KM110-R infection, was likely to be affecting ICP0 mutant replication. We found that although the levels of ISG 56K mRNA had dissipated by four days following induction, there was still a sufficient antiviral response to inhibit wild type virus. In subsequent experiments we used a higher MOI of the ICP0 mutant in order compensate for its deficiencies in overcoming an antiviral response.

An accurate determination of the proportion of cells harbouring the KM110-R genome is essential for drawing meaningful conclusions from our experiments. This was achieved using FACS analysis to measure the expression of red fluorescence expressed from KM110-R following its reactivation by SI with wild type virus. We were initially concerned that progeny KM110-R produced following reactivation of the quiescent genome could infect neighbouring cells, and therefore lead to an overestimate of the proportion of cells initially infected by KM110-R. We therefore tested the ability of wild type and IE mutant viruses to prevent subsequent infection of cells with progeny virus. We found that previous infection with wild type virus (Figure 4.2) or an ICP0, ICP22, or ICP27 mutant (data not shown) completely prevents SI of cells, and that ICP4 mutants were able to prevent SI of many, but not all cells (data not shown). The ability of HSV to prevent SI is attributed to the production of gD and its expression on the cell surface during productive infection (Campadelli-Fiume *et al.*, 1988, Campadelli-Fiume *et al.*, 1990, Dasika & Letchworth, 2000). As ICP4 mutants are unable to produce gD on non-complementing cell lines (DeLuca *et al.*, 1985), such as the HEL cells used in these experiments, it is unclear why ICP4 mutants were able to prevent SI in any of the cells. However, gD is a component of ICP4 mutant virions. Therefore, at the high MOI used in these experiments, many of the cells may have acquired enough gD to prevent superinfection from the fusion of virion and cellular membranes. Alternatively, these results may indicate that there is more than one mechanism for HSV to prevent reinfection.

We next asked if ICP0 mutants are able to overcome the antiviral response induced by KM110-R and infect cells that harbour the KM110-R genome. FACS analysis allowed us to determine the proportion of cells harbouring the quiescent KM110-R genome and the proportion of cells SI with the green fluorescent-tagged ICP0 mutant. We found that ICP0 mutants are capable of infecting cells that harbour KM110-R. However, the expression of the IE gene EGFP does not indicate that ICP0 mutants are able to overcome the antiviral response and complete the lytic cycle. We therefore used indirect immunofluorescence in order to determine if ICP0 mutant infection proceeds past the IE stage and leads to the formation of viral DNA replication compartments. We found that ICP0 mutants were able to form viral DNA replication compartments in cells that harboured the KM110-R genome. Results from Southern, Western and progeny assay confirmed that the ICP0 mutant was able to replicate in cells that harboured the quiescent KM110-R genome, but that it did not lead to reactivation of KM110-R. These results show that, in the absence of ICP0, the quiescent KM110-R genome is silent in the presence of an almost identical, replicating virus.

The experiments described in this investigation, in conjunction with previous work, indicate that the quiescent genome has been made inaccessible to cellular transcription and viral DNA replication machinery. Despite the presence of all the necessary components, there is no activation of promoters or replication of DNA from the quiescent genome. This is a surprising finding because DNA from other sources, such as transfected plasmids, is readily expressed in the presence of the necessary transactivators. The silencing of quiescent viral genomes could occur in at least two ways. The viral genome could be sequestered in an inaccessible place, or, non-exclusively, could be converted to an inaccessible state. Neither of these possibilities has been sufficiently researched to reveal how silencing is achieved.

There are many unanswered questions about quiescence, and therefore many areas of interest for further research. A few key topics will be discussed in detail here. First, both the location of and the structure of the quiescent genome are largely unknown. Additionally, the mechanism of action of ICP0 in the reactivation from and prevention of quiescence has not yet been determined. Lastly, the importance of the antiviral response for the establishment of quiescence has not been shown.

4.2 Location of the quiescent genome:

The precise cellular location of quiescent HSV-1 genomes is currently unknown; however, previous research indicates that they are likely to be found within the nucleus. Firstly, the genome of HSV-1 localizes to the periphery of nuclear domain 10 (ND10) structures following infection (Maul *et al.*, 1996). In these experiments, Maul et al used fluorescent in situ hybridization (FISH) to detect the viral genome and antibodies to PML and Sp100 to detect ND10 structures. Localization of the viral genome to the periphery of ND10 was found to occur even when viral gene expression was prevented using cycloheximide (Maul *et al.*, 1996); conditions that mimic what would be experienced by viruses that are unable to express IE genes and therefore become quiescent. Secondly, biochemical fractionation experiments performed by Jamieson et al show that by four days post-infection quiescent *in*1883 (VP16$^-$/ICP0$^-$/TK$^-$) genomes are found exclusively in the nuclear fraction (Jamieson *et al.*, 1995).

A number of techniques could be used to determine the location of quiescent HSV-1 genomes in relation to subnuclear structures. One method would be to use FISH in conjunction with antibodies to PML and Sp100, as was done by Maul et al, in order to determine the location of quiescent HSV-1 genomes in relation to ND10. Some disadvantages associated with this technique are that the high GC content of HSV makes FISH difficult, and that the cells must be fixed in order to perform FISH and to stain with antibodies: which can lead to artifacts. Another method, designed by Sourvinos and Everett, could be adapted to allow the detection of viral genomes in live cells with a fluorescent-labeled DNA binding protein (Sourvinos & Everett, 2002). Using yellow fluorescent protein (YFP) fused to the tetracycline repressor DNA binding protein, they were able to determine the location of plasmids marked with copies of the tetracycline operator sequence. In addition to this, they were able to detect ND10 structures in live cells using PML expressed from baculovirus (Sourvinos & Everett, 2002). An important advantage of this method is that, as it can be used in live cells, it would allow one to observe the location of the quiescent genome, in the same samples, over a period of time. However, there are also possible disadvantages. One is that the fluorescent-labeled DNA binding protein targeted to the viral genome could interfere with the establishment of quiescence which, as will be discussed later, likely involves the association of the viral genome with cellular proteins. Alternatively, cellular proteins involved in silencing the viral genome may prevent the fluorescent-labeled DNA binding proteins from binding the viral genome; therefore, the quiescent genomes would be undetectable by this method. In addition to their relative location in relation to ND10 structures, it would also be of interest to determine whether quiescent genomes localize to nuclear areas associated with high gene activity (euchromatin), or areas associated with silent genes (heterochromatin). DAPI staining of nuclear DNA, in conjunction with FISH or fluorescent-labeled DNA binding proteins, could be used to determine the location of quiescent genomes in relation to heterochromatin areas, which stain brightly with DAPI, or euchromatin areas, which stain less brightly (Maison *et al.*, 2002).

The techniques described above could be used to determine the location of quiescent genomes over the period of a number of days. This would allow us to determine if the location of the viral genome changes in accordance with the differences seen in its susceptibility to various transactivators over time. As previously mentioned in the introduction, the viral genome of quiescent mutants can initially be activated by the provision of VP16 or HMBA but, after 24 hours, the genome is immune to these activators and can be reactivated only by the provision of ICP0 or TSA (Everett *et al.*, 1998b, Harris & Preston, 1991, Preston & Nicholl, 1997, Samaniego *et al.*, 1998, Stow & Stow, 1989). It seems likely that, initially, the genome of quiescent mutant viruses localizes to areas adjacent to ND10. The experiments done by Maul et al (described above) showed that, at 5 hrs post-infection and in the presence of cycloheximide, the viral genome localizes to the periphery of ND10 (Maul *et al.*, 1996). Whether the viral genome is retained at these regions after it has become immune to activators such as VP16 and HMBA remains to be seen.

Movement of the viral genome away from ND10 structures once quiescence has been firmly established could indicate that sequestration of the viral genome away from components found in ND10 plays a role in its silencing. Sourvinos and Everett found that the association of the HSV-1 genome with ND10 structures is facilitated by viral sequences and proteins, and that genomes associated with ND10 have an increased probability of initiating DNA replication (Sourvinos & Everett, 2002). The genome of another member of the herpesvirus family, Epstein-Barr virus, is not associated with ND10 structures during latency (Bell *et al.*, 2000). However, following reactivation, there is an increased association of viral genomes with ND10 structures, and replication occurs in close association with the remnants of disrupted ND10 (Bell *et al.*, 2000). These experiments indicate that association with ND10 structures is advantageous for viral replication. Alternatively, continued association of the HSV-1 genome with ND10 structures once quiescence is firmly established may implicate ND10 components in the establishment of and maintenance of quiescence (discussed further below).

Movement of the viral genome to regions of cellular heterochromatin (a highly condensed form of DNA) once quiescence has been firmly established would imply that a close proximity to heterochromatin plays a role in the silencing of the viral genome. Genes that reside in heterochromatic regions such as the inactive X-chromosome, centromeric regions, and telomeres are not expressed (Elgin, 1996). Even genes close to heterochromatin can be silenced: a phenomenom first described in *Drosophila melanogaster*, and called position effect variegation (Eissenberg *et al.*, 1990, Eissenberg *et al.*, 1992). Alternatively, if the viral genome remains in areas of euchromatin (a less condensed form of DNA) once quiescence has been firmly established, it would imply that the mechanism for silencing the quiescent genome is maintained even in areas where other genes are highly expressed. This would indicate that the structure of the quiescent genome plays a very important role in its silencing.

4.3 Structure of the quiescent genome:

The structure of quiescent HSV-1 genomes and the cellular proteins involved in their silencing are currently unknown. Higher order chromatin structure is an important control of gene expression that can lead to the accessibility or inaccessibility of DNA to transcription factors and regulatory proteins. Histone and DNA modifications can lead to a more or a less compact structure by altering how tightly the DNA is bound by histones. These modifications can include DNA methylation of the dinucleotide CpG, and acetylation, phosphorylation, and methylation of histones (Marmorstein & Roth, 2001).

Heterochromatin is an extreme example of transcriptionally inert DNA. Markers of heterochromatin include; DNA methylation at CpG dinucleotides (Bird, 2002), hypoacetylation of histones (Jeppesen *et al.*, 1992), histone H3 methylation at lysine (K) 9 (Peters *et al.*, 2001) and enrichment in heterochromatin protein-1 (HP1) (James & Elgin, 1986).

The available data indicates that quiescent genomes may be packaged as heterochromatin. As previously mentioned in the introduction, all promoters tested to date, including those from HSV-1, HCMV, and human cells, are silent in the context of the quiescent genome (Preston & Nicholl, 1997, Samaniego *et al.*, 1998). This indicates that the repression of the quiescent genome is global and not likely sequence specific (Preston, 2000); which points to an epigenetic mechanism, such as heterochromatin, of silencing. Additionally, the histone deacetylase (HDAC) inhibitor trichostatin A (TSA) induces reactivation of quiescent d109 (mutant in all 5 IE genes) genomes (Hobbs & DeLuca, 1999). Exposure to TSA, which leads to histone hyperacetylation (Yoshida *et al.*, 1990), can disrupt HP1 proteins concentrated in heterochromatic areas (Taddei *et al.*, 2001). Therefore the de-repression of quiescent genomes in response to TSA also suggests a heterochromatin-based silencing mechanism.

A number of techniques could be used to test if the structure of quiescent genomes is similar to heterochromatin. For example, bisulfite sequencing, a method where unmethylated cytosines are chemically converted to uracils, could be used to test for the methylation at CpG dinucleotides that is commonly seen in heterochromatin. Kubat et al used this method to test for DNA methylation of latent genomes, but did not find methylation at any of the tested loci (Kubat *et al.*, 2004). Finding that quiescent genomes are DNA methylated could indicate structural differences between quiescent and latent genomes. The repression of latent genomes appears to be less severe than for quiescent genomes (Preston, 2000), therefore a different structure or silencing mechanism seems likely.

Proteins involved in the silencing of quiescent viral genomes could be identified using chromatin immunoprecipitation (ChIP) assays. Kubat and colleagues recently used this technique with an anti-acetyl histone H3 (K9, K14) antibody to identify regions of latent genomes associated with histone H3 acetylated at lysines 9 and 14 (characteristic of euchromatic or nonrepressed DNA) from areas that were not (Kubat *et al.*, 2004). Antibodies to cellular proteins known to be involved in transcriptional repression, such as HP1 and Sp100 (Seeler *et al.*, 1998), as well as antibodies to the various histone modifications known to mark heterochromatin, could be used to test the association of these proteins with quiescent genomes. However, the success of ChIP, and therefore the amount of information that can be gathered with this method, is very dependent upon the quality of the antibodies available. For example, antibodies that are of sufficient quality for ChIP are not yet available for trimethyl histone H3 (K9), a modification that recruits HP1 (Kubat *et al.*, 2004).

A technique developed by van Steensel and Henikoff may prove a useful method for determining if HP1, or other DNA binding proteins, are associated with quiescent genomes. They constructed a fusion protein consisting of Dam methylase linked to the amino terminus of full length HP1 and found that it could be used to determine the genes targeted by HP1 (van Steensel & Henikoff, 2000). When the Dam/HP1 fusion protein is expressed in cells it targets natural HP1 binding sites, which are then Dam methylated. Dam methylation leads to 6-methyl-A, which is not normally found in eukaryotic DNA. Methylated sequences can be distinguished from unmethylated sequences by comparing the restriction digest patterns of the enzymes *Dpn*I, which does not cleave methylated sites, and *Dpn*II, which does not cleave unmethylated sites. Van Steensel et al found that, in *Drosophila melanogaster*, the predominant targets of HP1 were pericentric genes and transposable elements (mostly in pericentric heterochromatin) (van Steensel *et al.*, 2001). Pericentric heterochromatin is an example of constitutive heterochromatin that remains condensed throughout the cell cycle (Maison *et al.*, 2002). An association between HP1 and quiescent genomes could indicate that the virus is targeted by a cellular system used for permanent gene silencing.

Determination of the structure of quiescent genomes will give important clues for the elucidation of the cellular repression machinery, as well as the mechanism for the ICP0-mediated defense against this repression.

4.4 Role of ICP0 in the prevention of and the reactivation from quiescence:

4.4.1 Prevention of quiescence:

The way in which ICP0 prevents the repression of the viral genome is still unknown. The discovery of the E3 ubiquitin ligase activity of ICP0 (Everett *et al.*, 1993, Freemont *et al.*, 1991) has lead to speculation that ICP0 leads to the degradation of a cellular repression mechanism through the ubiquitin-proteasome pathway. However, the identity of this putative cellular repression mechanism is also unknown. One possibility is that ICP0 disrupts ND10 structures (Everett & Maul, 1994, Maul & Everett, 1994, Maul *et al.*, 1993) in order to prevent repression. A number of ND10 components, including PML, Sp100, HP1, and hDaxx have been shown to be transcriptional repressors (Hollenbach *et al.*, 2002, Seeler *et al.*, 1998, Wu *et al.*, 2001), supporting the viewpoint of ND10 as an intra-nuclear defense mechanism protective against viral infection. Therefore, the HSV-genome, as well as the genomes of other DNA viruses, could be vulnerable to these repressors unless they are degraded or dispersed. DNA viruses such as HSV, HCMV, EBV, adenovirus, and papillomavirus whose genomes localize to areas adjacent to ND10 upon infection all have proteins that lead to the degradation or alteration of ND10-proteins (reviewed in (Everett, 2001)).

Both PML (Everett *et al.*, 1998a) and Sp100 (Chelbi-Alix & de The, 1999) are targeted for degradation by ICP0. Additionally, the disruption of ND10, which occurs as a result of the ICP0-mediated degradation of PML (Ishov *et al.*, 1999, Zhong *et al.*, 2000), may cause the dispersal of other components involved in transcriptional repression, and therefore prevent them from interacting with the viral genome. However, the importance of this is unclear as overexpression of PML, which prevents the dispersal of ND10 components following exposure to ICP0, does not have an inhibitory effect on HSV-1 (Lopez *et al.*, 2002). ND10 may not be the only structure disrupted by ICP0 in order to disperse transcriptional repressors. HP1 and hDaxx are also components of centromeres, which are disrupted by the ICP0-mediated degradation of the centromeric proteins CENP-C and CENP-A (Everett *et al.*, 1999a, Lomonte & Everett, 1999). Experiments to determine additional proteins that are targeted for degradation by ICP0 could lead to identifying the cellular repression mechanism. Proteins suspected of being involved could be tested by Western analysis. Any that are degraded following infection with wild-type, or a virus that expresses only ICP0, but are not degraded following infection with an ICP0 mutant are likely to be targets of ICP0. Once more is known about the structure of the quiescent genome, proteins that are involved in the formation of that structure could be tested to determine if they are targets of ICP0.

4.4.2 Reactivation from quiescence:

Provision of ICP0 is sufficient to reverse repression and lead to reactivation of quiescent genomes (Everett *et al.*, 1998b, Harris & Preston, 1991, Stow & Stow, 1989); however, the mechanism for this is unknown. Other agents that are able to reverse repression, such as TSA and ICP0 functional homologues from other herpes viruses, have been shown to inhibit HDACs. For example, the mouse cytomegalovirus (MCMV) iE1 protein recruits HDAC-2 to ND10. The binding of HDAC-2 by MCMV iE1 decreases its deacetylation activity and thereby reverses its repressive effect (Tang & Maul, 2003). Similarly, the interaction between bovine herpesvirus 1 immediate-early protein (bICP0) and HDAC1 leads to activation of transcription and inhibition of transcriptional repression (Zhang & Jones, 2001). This raises the possibility that the ability of ICP0 to reverse repression of quiescent genomes may also involve inhibition of HDACs. However, a mechanism by which ICP0 inhibits HDAC1 and 2 is not likely to be similar to the examples just described, as it has been shown that ICP0 does not co-immunoprecipitate with either of these HDACs (Lomonte *et al.*, 2004). Additionally, the proteasome inhibitor MG132 has been shown to prevent ICP0-mediated reactivation of quiescent genomes (Everett *et al.*, 1998a), indicating that proteasomal degradation of a cellular factor is necessary.

ICP0 may have a direct role in the inhibition of HDACs. For example, HDAC1 levels have been shown to decrease in response to HSV-1 infection (Poon *et al.*, 2003). Alternatively, ICP0 may have an indirect role by leading to the degradation of a cellular factor that leads to the activation of, or prevents the inactivation of HDACs. Poon et al found that HDAC1 and 2 were modified by the viral protein kinase U_S3 in HSV-1 infected cells in what appeared to be an ICP0 dependent manner (Poon *et al.*, 2003); however, it is still unknown if this modification affects the function of these HDACs. The effect of ICP0 on the deacetylase activity of HDACs could be determined. HDACs immunoprecipitated from uninfected samples and samples infected with wild type virus, an ICP0 mutant, and an U_S3 mutant could be assayed for deacetylase activity using the same in vitro assay as Tang et al, with ^{3}H-acetylated histone 4 as the substrate (Tang & Maul, 2003). A decrease in the deacetylation ability of the HDACs in response to wild type infection, but not ICP0 or U_S3 mutant infection, could indicate that the modification of these HDACs by U_S3 decreases their deacetylation activity and that ICP0 is somehow necessary for this modification to occur. A possible role for ICP0 could be to lead to the degradation of a cellular factor that prevents U_S3 from modifying these HDACs. However, as ICP0, in the absence of any other HSV gene product, is sufficient for the reactivation of quiescent genomes (Everett *et al.*, 1998b, Harris & Preston, 1991, Stow & Stow, 1989), it is unlikely that U_S3 would be required for the main mechanism by which ICP0 reverses repression.

4.5 Role of the antiviral response in the establishment of quiescence:

The importance of an antiviral response for the establishment of quiescence is currently unknown. As described previously in the introduction, HSV infection triggers an antiviral response that leads to the induction of many of the same ISGs as IFN (Mossman *et al.*, 2001). This antiviral response is disarmed both by ICP0, which overcomes a barrier to transcription, and by ICP34.5, which overcomes a barrier to translation (Mossman & Smiley, 2002). However, viruses that establish quiescence, such as the VP16/ICP0 mutant KM110, do not express viral proteins and therefore cannot disarm the antiviral response (Mossman *et al.*, 2001). The antiviral response triggered following viral infection may up-regulate the expression of proteins involved in silencing the quiescent genome. For example, over-expression of Sp100 (an ISG (Regad & Chelbi-Alix, 2001)) leads to greater accumulation of HP1 in ND10 (Seeler *et al.*, 1998). If HP1 is involved in silencing of the viral genome, this could indicate how the establishment of quiescence is facilitated by the induction of an antiviral response.

A convenient system for determining if the establishment of quiescence is hindered or prevented in the absence of an antiviral response would be to use mouse embryonic cells. If mutant viruses such as KM110 establish quiescence in mouse embryonic cells, cell lines with specific genes knocked out could be used to test the importance of each gene for the silencing of viral genomes. For example, Collins et al found that the induction of ISGs in response to HSV infection requires IRF-3 (Collins *et al.*, 2004). Therefore, if an $IRF3^{-/-}$ mouse cell line supported the growth of KM110, it would indicate that an antiviral response is necessary for the establishment of quiescence. The effect of knocking out various ISGs, such as PML or Sp100, could be tested in a similar manner. Use of mouse embryonic cells would also open up the possibility of determining if Toll-like receptor 3 (TLR3) signaling, and subsequent activation of IRF-3 (Fitzgerald *et al.*, 2003), is responsible for the induction of an antiviral response following infection by enveloped viruses.

U2OS cells, which are permissive for VP16/ICP0 mutants (Yao & Schaffer, 1995), do not show induction of ISG 56K in response to KM110 infection (Mossman & Smiley, 2002). This raises the possibility that the inability of U2OS cells to mount an antiviral response in response to KM110 is responsible for their permissive phenotype. However it is also possible, as suggested by Yao and Schaffer, that U2OS cells express a protein with similar properties to ICP0 that allows them to complement VP16/ICP0 mutant viruses (Yao & Schaffer, 1995). These possibilities can be distinguished by determining if the complementation of KM110 on U2OS cells results from a gain of function, which would be a dominant phenotype, or a loss of function, which would be a recessive phenotype. HEL cells, which are non-permissive for KM110, could be fused to U2OS cells. The fused cells could then be infected and tested for their ability to support growth of KM110. A permissive (dominant) phenotype would indicate that U2OS cells have a protein that complements VP16/ICP0 mutant viruses. A non-permissive (recessive) phenotype would suggest that the ability of U2OS cells to support KM110 is a result of a mutation that prevents them from silencing the mutant viral genome. This could be the result of a mutation that inactivates a signaling pathway necessary for the induction of an antiviral response following HSV infection. Alternatively, the permissive phenotype of U2OS cells may be a result of a mutation that inactivates the putative gene silencing mechanism.

4.6 Concluding remarks:

At this point, a great deal of work will need to be done in order to determine how cells repress mutant HSV genomes, and how the wild type virus is able to avoid this repression. The answers to these questions will have broader implications than simply a greater understanding of HSV biology. Increased knowledge about cellular mechanisms for fighting HSV and other DNA viruses could eventually lead to new treatments or cures. Viral vectors for use in gene therapy may be vulnerable to the same cellular mechanism that is responsible for the suppression of quiescent HSV mutants. Therefore, information learned from studies on quiescence could also be used to overcome difficulties encountered in the development of gene therapy vectors.

CHAPTER 5 : REFERENCES

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